# STUDIES ON SOME FREE AND CONJUGATED STEROIDS IN URINE.

by

Elisabeth Sinclair Sutherland, B.Sc.

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# SECTION I.

## General Introduction

Studies on the steroids excreted in the urine have played a fundamental part in building up the present knowledge of sex hormone metabolism. The isolation of some of the quantitatively more important urinary steroids preceded, and facilitated, the isolation of the primary hormones from gonadal sources. In the two decades which have passed since the first crystalline steroid was isolated from urine, a considerable amount of information has been acquired on the nature of the steroids excreted by normal individuals.

Following on the observations of Butler and Marrian (1937), Burrows, Cook, Roe and Warren (1937) and others, on the steroid excretion in cases where the adrenal cortex was hyperactive, and of Callow and Callow (1940) and Hirschmann (1940) on the 17-ketosteroid excretion of male and female castrates, it became obvious that the adrenal cortex, as well as the gonads, contributed to the urinary steroids. Several groups of workers have studied the steroids excreted/

excreted in the urine in cases of hyperplasia or tumour of the adrenal cortex, but although this work has led to the isolation of a number of new compounds, very little light has been thrown on the routes of steroid metabolism. Nor have such studies proved so far to be of differential diagnostic value: the steroid excretion picture varies so greatly from patient to patient, that no conclusions as to the nature of the hyperactivity can be drawn.

The apparent diversity of steroid excretion in these pathological conditions may be due, in part, to the small number of cases thoroughly investigated, and to the different, and imperfect, techniques of hydrolysis and isolation used. While isolation techniques have now reached a very high level of perfection (Dobriner, Lieberman and Rhoads, 1948), the introduction of specific and accurate estimation techniques is overdue for many of the urinary steroids, since isolation procedures, however good, can never be regarded as quantitative, and require a considerable expenditure of time and labour.

It must be admitted, however, that the study of urinary steroids can give but a limited knowledge of metabolic/

metabolic processes, since it is well known that the recovery of administered steroids as recognisable metabolic products in the urine is not usually more than 10-20%, and often less. It is unlikely that losses during hydrolysis and isolation procedures represent more than a small fraction of the un-accounted for material. For this reason the more direct approach to the problems of steroid metabolism which has recently been initiated in the introduction of the tissue slice technique, e.g. Samuels, McCaulay and Sellers, 1947; Clark, Kochakian and Lobotsky, 1947) is of particular interest, as are also the results reported on the excretion of steroids in bile (e.g. Pearlman and Cerceo, 1948; Pearlman and Rakoff, 1949). Nevertheless, practically all the definite knowledge we at present possess of steroid metabolism has been acquired by studying the excreted products following massive administration experiments.

At one time it appeared as if the useful knowledge to be gained from the study of the urinary steroids had been practically exhausted by the intensive investigation which this subject has undergone. The discovery of the presence in urine of substances possessing cortin activity (Weil and Browne/

Browne, 1939; Dorfman, Horwitt and Fish, 1942), followed by the isolation of some of the more highly oxygenated steroids, has opened up a whole new field of research. Since many of these compounds are acid-labile, the recent introduction of the use of enzymic methods for the hydrolysis of steroid conjugates is of great importance. In addition, estimation results reported following enzymic hydrolysis (Cohen and Bates, 1949; Buehler, Katzman, Doisy and Doisy, 1949) suggest that even in the case of the oestrogens and neutral 17-ketosteroids interesting discoveries may yet remain to be made.

It seems certain that enzymic hydrolysis procedures will be much used in the future: possibly the day will come when the quantitative results obtained following acid hydrolysis will be treated with as little regard as is at present given to results obtained before such hydrolysis procedures were employed. In the past, the study of the conjugated steroids has been neglected, possibly because of the difficulties experienced in purifying these compounds, compared with the free steroids. The specific nature of enzyme hydrolysis obviously makes a more extensive knowledge of this subject desirable, and even necessary.

Using/

Using improved techniques of hydrolysis and estimation, studies on urinary steroids may still be expected to make important contributions to the knowledge of steroid metabolism.

The work presented in this thesis is divided into five sections, two of which (Sections II and V) are devoted to attempts to devise estimation techniques for the free steroids, pregnane-3$\alpha$:17$\alpha$:20$\alpha$-triol and pregnan-3$\alpha$-ol-20-one. Sections III and IV deal with the isolation of conjugated steroids, one of which, sodium pregnane-3$\alpha$:20$\alpha$-diol glucuronidate, has previously been isolated in an impure form; the other, sodium pregnan-3$\alpha$-ol-20-one glucuronidate, has been isolated for the first time. The final section (VI) is concerned with experiments on the hydrolysis of sodium pregnanediol glucuronidate with ox-spleen $\beta$-glucuronidase.

## SECTION II.

## THE ESTIMATION OF PREGNANE-3α:17α:20α-TRIOL.

A. Introduction

1. The isolation of pregnane-3α:17α:20α-triol.
2. The biological formation of pregnanetriol.
3. The estimation of urinary steroids in pathological conditions and the significance of such results.

B. Experimental and Results

1. Oxidation of pregnanetriol with lead tetraacetate.
2. Oxidation of pregnanetriol with periodic acid.
3. The estimation of 'pregnanetriol-like' compounds:
   (a) in normal pregnancy urine.
   (b) in urine from cases in which the adrenal cortex was believed to be hyperactive.
   (c) in the urine of normal subjects receiving large doses of progesterone.
   (d) Discussion of these results.

C. Summary.

1. The isolation of pregnane-3α;17α;20α-triol.[x]

Pregnane-3α;17α;20α-triol, isolated by Butler and Marrian in 1937 from the urine of two women with adrenal hyperplasia, was the first abnormal steroid to be isolated from the urine of cases in which a pathological hyperactivity of the adrenal cortex existed. Its isolation led Butler and Marrian to suggest that such urines might prove a rich source of other interesting steroids; the suggestion was amply confirmed, and this has been one of the most active lines of steroid research during the past ten years.

Pregnanetriol/

x The compounds referred to on this and the following page were variously reported as possessing the C 17 hydroxyl group in the α- or β-position, or without any reference to the configuration at C 17. Since von Euw and Reichstein (1947) have shown that the side chain in the adrenal steroids is β-oriented, whether they possess a tertiary hydroxyl group at C 17 or not, and thus the hydroxyl group is always α-oriented, the urinary steroids are presumed to have the same configuration (Fieser and Fieser, 1948).

Pregnanetriol was also the first urinary steroid possessing a C 17 tertiary hydroxyl group to be isolated. Although this configuration is found in three of the cortical hormones, as well as in many of the accompanying inactive steroids, it is not common in urinary steroids. Only four other compounds of this nature have been isolated from urine; and three of these, like pregnanetriol, have only been found in pathological urines. Pregnane-3α:17α-diol-20-one has been isolated by Lieberman and Dobriner (1945) from four abnormal cases, and $\Delta^5$-pregnene-3β:17α-diol-20-one by Hirschmann and Hirschmann (1947) from the urine of a boy suffering from adrenocortical carcinoma. 17-Hydroxycorticosterone has been isolated by Mason and Sprague (1948) from a case of Cushing's syndrome; and from patients with rheumatoid arthritis receiving adrenocorticotrophic hormone, by Mason (1950).

The only 17-hydroxy C 21 steroid which has been isolated from normal urine is 17-hydroxy-11-dehydrocorticosterone which Schneider (1950) has isolated in small amounts from the urine of normal men/

men.

Pregnane-3α:17α:20α-triol has also been isolated from the urine of three cases of adrenal hyperplasia and one of adrenal cortical tumour, by Mason and Kepler (1945). These workers showed that pregnanetriol can be excreted conjugated as the glucuronide: by the Venning (1937) method for isolation of sodium pregnanediol glucuronidate, they were able to isolate a mixed glucuronidate, which, on enzymic hydrolysis, gave mainly pregnanetriol and pregnane-3α:20α-diol.

The acid hydrolysis techniques routinely used for the investigation of urinary steroids in most laboratories may be responsible for the failure of other groups of workers to isolate pregnanetriol. Talbot and Eitingon (1944) demonstrated that in two cases of adrenocortical carcinoma, and in one case of adrenocortical hyperplasia, material was present in the neutral non-ketonic fraction which gave rise to 17-ketosteroids on periodic acid oxidation. This material could be demonstrated by hydrolysis with barium chloride, buffered at pH 6, or by hydrolysis with acetone-dried rat liver enzyme, but was apparently completely destroyed by acid hydrolysis. Butler/

Butler and Marrian originally isolated pregnanetriol from the ethereal extract of unhydrolysed urine concentrates, and it is probable that considerable bacterial hydrolysis of the urine had occurred before it was concentrated. This would explain the high yield of 'free' steroids encountered. It is true that Mason and Kepler isolated pregnanetriol from acid-hydrolysed urine, but it is perhaps relevant that the quantities isolated by them varied from 1.4 to 3 mg. per litre, while Butler and Marrian (1937, 1938) isolated 13, 16 and 40 mg. per litre from their cases, and Talbot and Eitingon estimated the non-ketonic 17;20-dihydroxy steroids in three cases to be between 10 and 16 mg. per litre.

2. The biological formation of pregnanetriol.

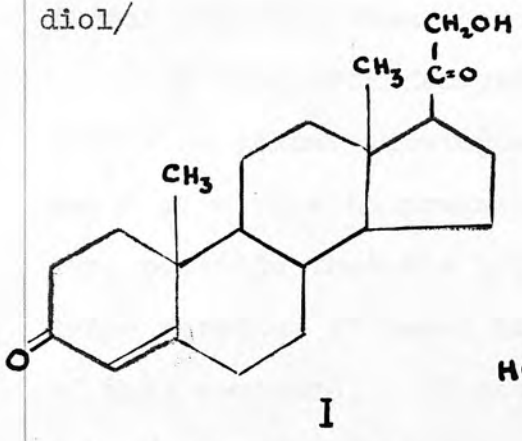
There is, as yet, no experimental evidence on the mode of formation or the metabolism of pregnanetriol. The fact that it has so far been isolated only from the urine of patients suffering from adrenal cortical hyperplasia, or having an adrenalcortical tumour, together with the presence of a tertiary hydroxyl group at C 17, suggests, very /

very strongly, that the adrenal cortex is its site of origin. As must be the case with such substances, found only in pathological urines, it is not known whether its presence in the urine is due to its formation by some completely abnormal metabolic process, or to excessive formation, or deficient utilization, or both, occurring in a normally established metabolic pathway.

On the basis of metabolism experiments with other steroids, 17-hydroxy deoxycorticosterone appears to be its most likely precursor. The excretion of pregnane-3α:20α-diol (II) following administration of deoxycorticosterone (I), or its acetate, has been reported by several groups of workers. Although in some of these cases (Cuyler, Ashley and Hamblen, 1940) identification of the end-product was not convincing, any precipitate obtained by the Venning (1937) method and melting within 30° of the correct melting point being considered as sodium pregnanediol glucuronidate, in other cases the pregnanediol was satisfactorily identified. Hoffman, Kazmin and Browne (1943) isolated pregnane-3α:20α-diol from the urine of rabbits to which they had/

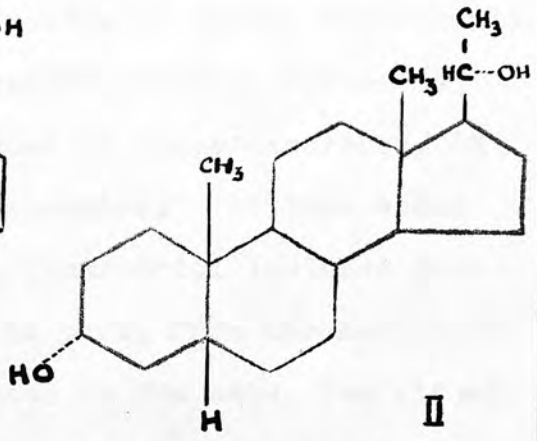
had administered deoxycorticosterone acetate, and Horwitt, Dorfman, Shipley and Fish (1944) reported similar results in human subjects and in chimpanzees. The pregnanediol isolated was equivalent to only 1-3% of the deoxycorticosterone administered (Horwitt et al., 1944), but it is well known that urinary recoveries of administered steroids are very low. Following oral administration of pregnanediol itself, less than 20% of the administered dose is excreted in the urine (Sommerville and Marrian, 1950).

The type of reaction involved in the conversion of deoxycorticosterone to pregnanediol is not uncommon in steroid metabolism. Progesterone, $\Delta^4$-pregnene-3:20-dione (III) also gives rise to pregnane-3α:20α-diol (Venning, Henry and Browne, 1937), and Mason (1948) has isolated pregnane-3α:20α-diol/




I

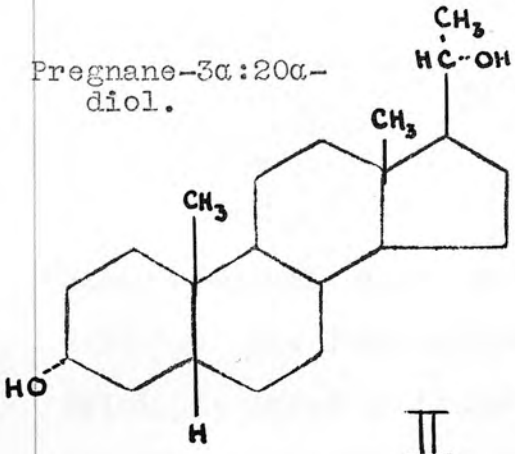
Deoxycorticosterone



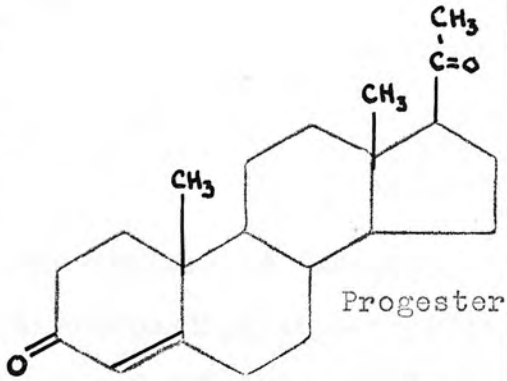

II

Pregnane-3α:20α-diol

Pregnane-3α:20α-diol.



II

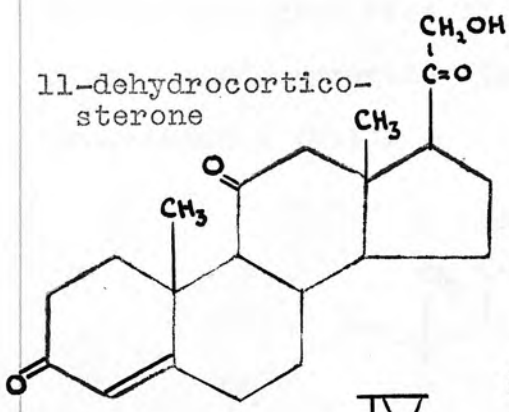


Progesterone

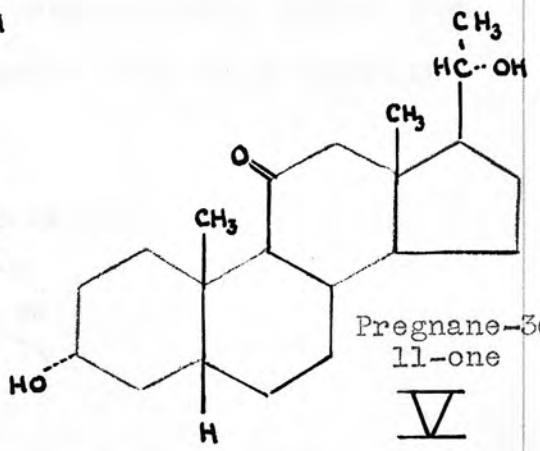
III

diol-11-one (V) from the urine of two Addison's disease patients receiving large doses of 11-dehydrocorticosterone (IV).

11-dehydrocorticosterone



IV



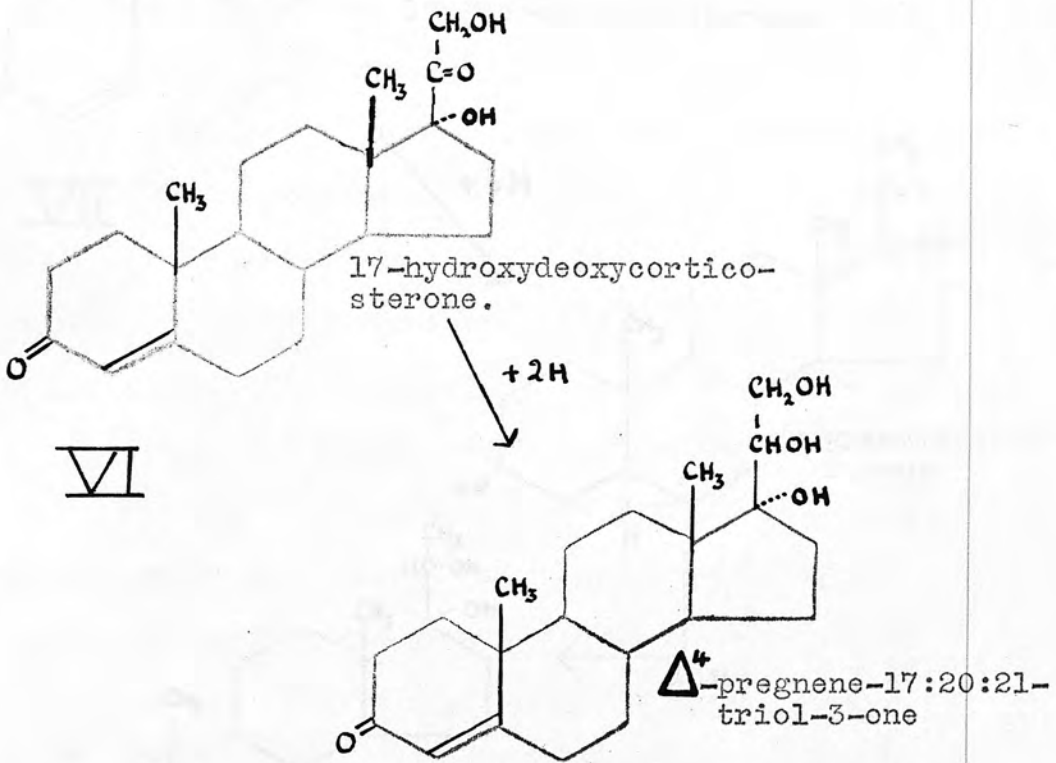
Pregnane-3α:20α-diol-11-one

V

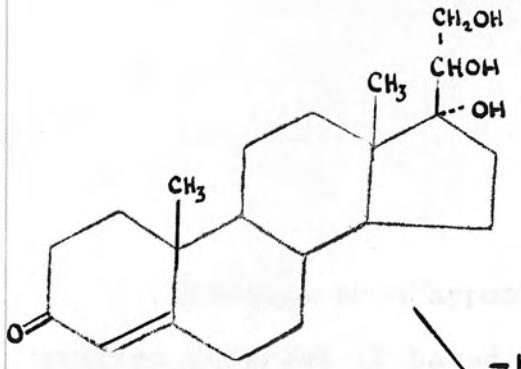
Lieberman (1947) has reported the isolation of the former compound from the urine of normal individuals.

If 17-hydroxydeoxycorticosterone follows a metabolic pathway analogous to those described, it would give rise to pregnanetriol. It thus seems very possible that the pregnanetriol isolated from urine results, at least in part, from the metabolism of this compound. If such is the case, two already known/

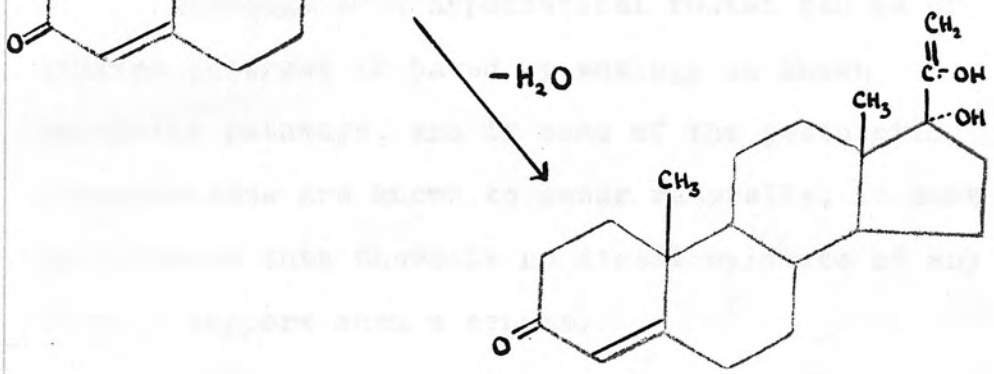
known compounds might be intermediate in the conversion: the C 20 ketone corresponding to pregnanetriol, isolated by Lieberman and Dobriner (1945), and 17α-hydroxyprogesterone, isolated from beef adrenals by Pfiffner and North (1941). The completely speculative scheme, represented diagramatically below, shows one of the series of reactions which might give rise to pregnanetriol (VIII) from 17-hydroxydeoxycorticosterone (VI) or 17-hydroxyprogesterone (VII).

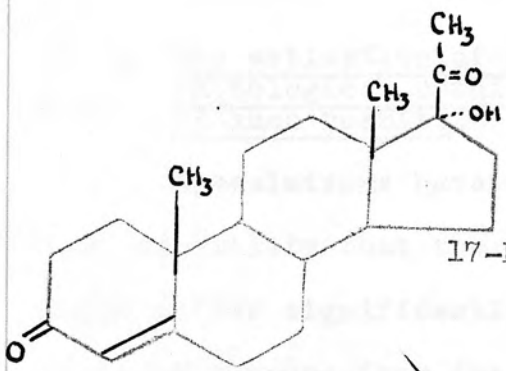



VI



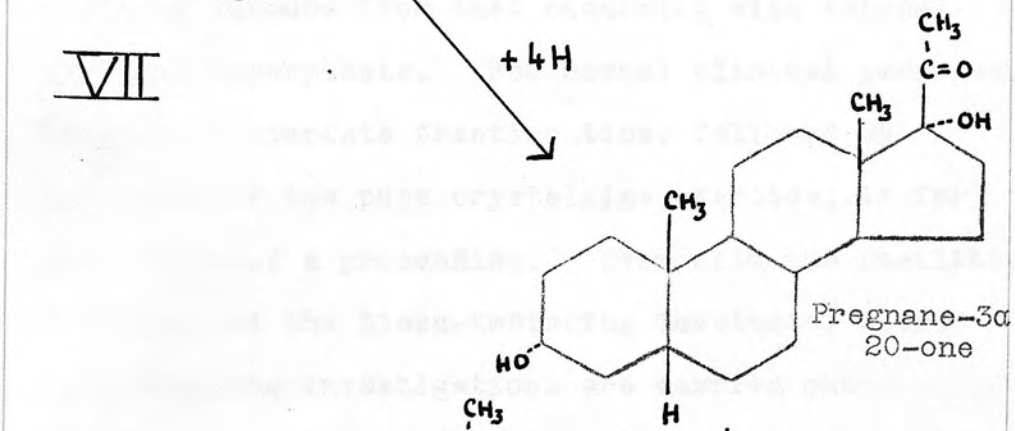
$\Delta^4$-pregnene-17:20:21-triol-3-one



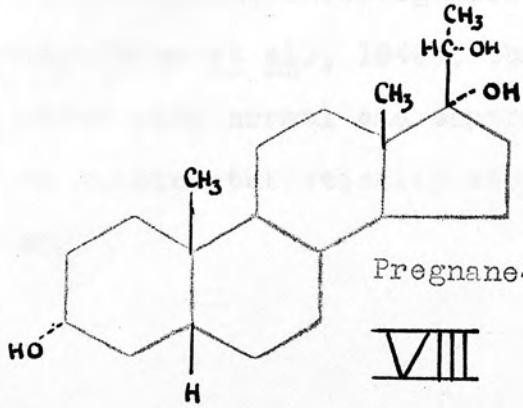


17-Hydroxyprogesterone

VII



Pregnane-3α:17α-diol-20-one



Pregnane-3α:17α:20α-triol

VIII

Although such hypothetical routes can be of limited interest if based by analogy on known metabolic pathways, and if some of the postulated intermediates are known to occur naturally, it must be stressed that there is no direct evidence of any kind to support such a scheme.

3. The estimation of urinary steroids in pathological conditions and the significance of such results.

Speculations have frequently been made on the possibility that the steroid excretion picture might differ significantly in cases of adrenal-cortical tumours from that occurring with adrenal-cortical hyperplasia. For normal clinical purposes, however, a complete fractionation, followed by isolation of the pure crystalline steroids, is far too laborious a proceeding. Even with the facilities available at the Sloan-Kettering Institute, where such complete investigations are carried out (Dobriner et al., 1948), the progress made is slow, since many normal and abnormal cases are necessary to obtain statistically significant data. Results so/

so far obtained suggest that while there may be significant differences in the steroid excretion picture when comparing hundreds of cases, the overlapping is such that the results are of little value in diagnosing individual cases. To quote Mason and Kepler (1945): 'If the presence of any compound in the urine could be established as characteristic of hyperplasia or of a tumor exclusively, a valuable tool for differential diagnosis would be available. When we consider the protean symptoms of adrenal-cortical tumors it seems very unlikely that such a compound will be discovered'.

Various methods have been evolved for studying the different patterns of 17-ketosteroid excretion; some involve fractionation, after separation of the ketonic fractions by the method of Girard and Sandulesco (1936), into alcoholic and non-alcoholic, digitonin precipitable and non-precipitable fractions - e.g. Talbot, Butler and MacLachlan (1940), Pincus and Pearlman (1941). The 17-ketosteroids present in each fraction are then measured by means of the m-dinitrobenzene colour reaction.

Other/

Other workers have used chromatographic methods of varying complexity for separating the neutral ketonic fraction. While chromatography has been widely used to facilitate the isolation of pure steroids, for example by Callow and Callow (1939) and Dobriner and his coworkers (1948), adaptations of this technique have been used for clinical determinations by Dingemanse, Huis in't Veld and de Laat (1946) and by Robinson and Goulden (1949). Using standard conditions, a number of eluates are collected separately, and their 17-ketosteroid content determined. Although this method appears superficially to be well suited to routine determinations, scrupulous care must be given to the standardization of the adsorbent material and the purity of the solvents, if reproducible results are to be obtained.

The study of the non-ketonic urinary steroids has been relatively neglected. Pregnane-3$\alpha$:20$\alpha$-diol is probably the only neutral, non-ketonic steroid which it is possible to estimate satisfactorily in urine (Sommerville, Gough and Marrian, 1948).

In view of the fact that pregnanetriol has only/

only so far been isolated from pathological urines, it was felt that it would be of interest to be able to detect and estimate it in urine, and thus eventually build up a body of evidence on the nature of the adrenal disorders which caused its excretion. The suggestion had originally been made that the excretion of pregnanetriol might be characteristic of adrenal hyperplasia; its isolation by Mason et al.(1945) from the urine of a patient with an adrenalcortical tumour shows that it is not associated specifically with this condition.

The work of Mason and Kepler does, however, suggest that the excretion of pregnanetriol is more often associated with non-malignant hyperactivity of the adrenal cortex, rather than with a carcinoma. According to these workers' investigations, although, as might be expected, there appears to be no clear-cut line between the steroids excreted in cases of hyperplasia and those excreted by tumour cases, there may be two main types of disturbance of steroid metabolism, one characterized by an extremely high dehydroepiandrosterone excretion level, and the absence/

absence of pregnanetriol, and the other by the excretion of pregnanetriol, and an only moderately elevated dehydroepiandrosterone excretion level.

A relatively simple method of estimating urinary pregnanetriol, preferably one which could be used for routine determinations in hospital laboratories, would lead to a much more satisfactory state of knowledge on the type of cases which do, or do not, excrete this compound. This information might be of interest in other pathological conditions besides those which have been referred to. Recent work suggests that an abnormality in steroid metabolism may be associated with rheumatoid arthritis and hypertension (Sommerville, 1950).

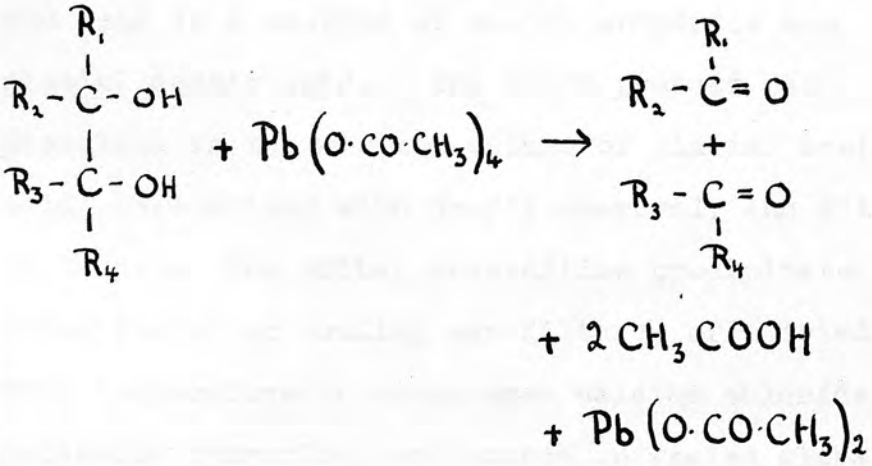
B. Experimental /

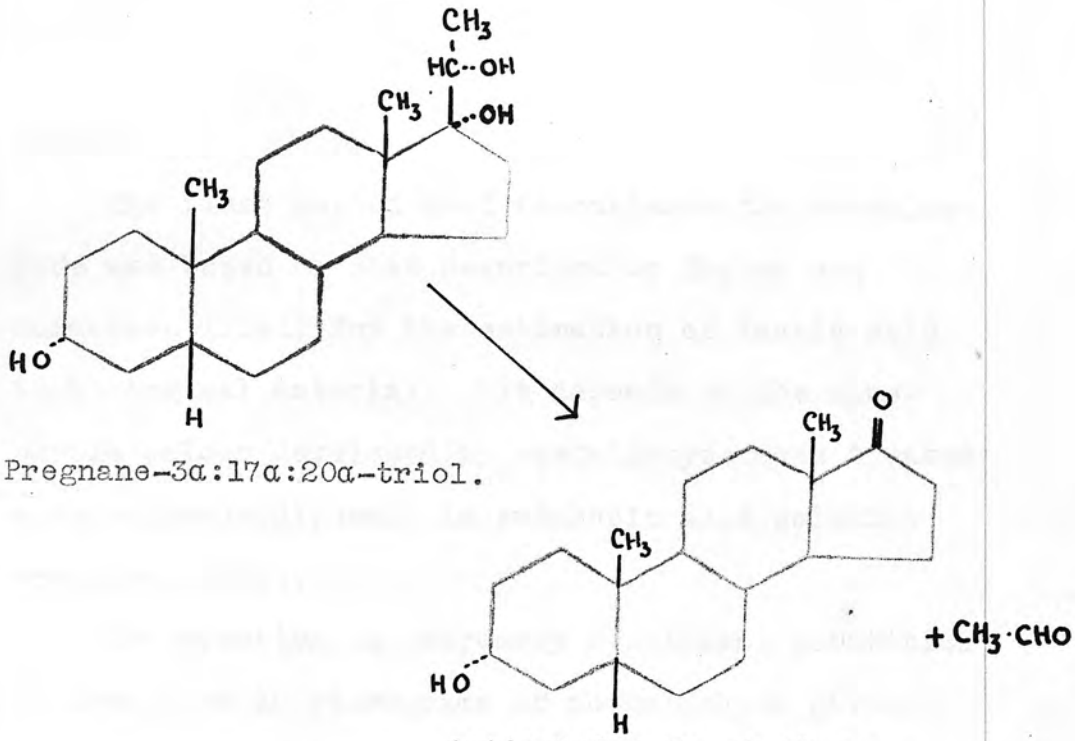
B. Experimental.

1. Oxidation with lead tetraacetate.

It was originally planned to estimate the pregnanetriol by oxidative fission of the side chain with lead tetraacetate, and measurement of the acetaldehyde so formed. This reaction was used by Butler and Marrian (1937) in the characterization of pregnanetriol.

The ability of lead tetraacetate to cause α-glycols to undergo quantitative oxidative fission was first discovered by Criegee (1931). The reaction is specific to α-glycols, but appears to be generally applicable within this group of compounds. The oxidation takes place according to the equations:






Pregnane-3α:17α:20α-triol.

Aetiocholan-3α-ol-17-one.

The lead tetraacetate was prepared according to the method of Oesper and Deasy (1939) by passing a slow stream of chlorine through a suspension of red lead in a mixture of acetic anhydride and glacial acetic acid. The crude product was dissolved in the minimum volume of glacial acetic acid, decolorized with Norit charcoal, and filtered while hot. The white, crystalline precipitate which formed on cooling was filtered off, dried at room temperature in vacuo over calcium chloride and potassium hydroxide, and stored in sealed glass tubes/

tubes.

The first method used to estimate the acetaldehyde was based on that described by Barker and Summerson (1941) for the estimation of lactic acid in biological material. It depends on the blue-purple colour developed by acetaldehyde when treated with p-hydroxydiphenyl in sulphuric acid solution (Eegriwe, 1933).

The reaction is extremely sensitive, quantities of from 1 to 10 micrograms of acetaldehyde giving intense colours in a total volume of 14 ml. of reaction mixture.

Recovery experiments using a dilute aqueous solution of acetaldehyde, showed that the acetaldehyde could be estimated quantitatively after 2½ hr. aeration, if trapped in a tube containing sulphuric acid and water in the proportions used for the colour reaction. Later experiments showed that unless aeration was started very slowly, it was preferable to have two such tubes in series. The procedure was as follows: A number of tubes ( 6 x ¾" ) were connected in series in the usual way for aeration/

aeration; each tube containing a dilute aqueous solution of acetaldehyde was followed by a receiver tube containing 2 ml. of water, 0.1 ml. of a 5% (w/v) solution of $CuSO_4.5H_2O$, and 12 ml. of conc. $H_2SO_4$, (A.R.), and a gentle stream of air was sucked through the aeration train. After 2½ hr. the tubes were disconnected and 0.2 ml. of the p-hydroxydiphenyl reagent (1.5% (w/v) in 0.5% (w/v) NaOH) was added to the receiver tubes, which were then kept at 30°C. for half an hour. They were then heated in a boiling water bath for 90 seconds, to dissolve the excess reagent, cooled, and the colour developed compared to a similarly treated reagent blank in the Spekker photoelectric absorptiometer using Ilford spectrum yellow-green (605) filters. On attempting to carry out estimations on known amounts of pure pregnanetriol, a recovery of about 50% of the theoretical amount of acetaldehyde was obtained after fifteen hours' aeration, using 10 mg. of triol in 5 ml. N/10 $Pb(OAc)_4$ in glacial acetic acid. The second trap, in this case, gave a very small positive value. Readings were made against the reagent blank, which was run at the same time as the estimation, the tubes being aerated in the following order:- (i) bisulphite trap/

trap to purify incoming air, (ii) lead tetraacetate in glacial acetic acid, (iii) sulphuric acid-water trap, (iv) lead tetraacetate in glacial acetic acid with pregnanetriol added, (v) and (vi) sulphuric acid-water traps.

It was at first thought that this low recovery might be due to inefficiency in the trapping of acetaldehyde. An alternative method involving distillation of the acetaldehyde into the sulphuric acid-water reaction mixture was devised, but low results were consistently obtained, and it was finally discovered that the presence of small amounts of acetic acid had an inhibiting effect on the p-hydroxy-diphenyl colour development.

Benzene and dioxane were considered as possible alternative solvents, but these also had an adverse effect on the colour reaction.

The idea of using this colour reaction was therefore given up, and instead, the possibility of trapping the acetaldehyde in bisulphite solution and then estimating it by iodometric titration was examined. The acetic acid solvent again introduced difficulties. During aeration sufficient acetic acid was carried over into the bisulphite to make the/

the solution strongly acidic, and no acetaldehyde was trapped. If a sodium bicarbonate trap was introduced between the reaction tube, and the tube containing bisulphite, most of the acetaldehyde was apparently retained in the bicarbonate solution.

The possibility of trapping the acetaldehyde in 2:4-dinitrophenylhydrazine solution, and measuring the insoluble hydrazine formed was next investigated. Acetaldehyde 2:4-dinitrophenylhydrazone was prepared and recrystallized to constant melting point (165-167° uncorr.). It was at first proposed to measure the hydrazone formed by means of the intense red colour which it gives in the presence of alcoholic potassium hydroxide. Over the desired range, however, the untreated ethanolic solution was sufficiently coloured, and since this eliminated all complications due to precipitation of carbonate from the alcoholic potassium hydroxide solution, this method was adopted. In a volume of 10 ml. ethanol, the yellow colour was found to be proportional to the concentration over the range 50-500 micrograms of acetaldehyde 2:4-dinitrophenylhydrazone. Readings were made with the Spekker photoelectric colorimeter using Ilford spectrum violet (601) filters/

filters. Qualitative tests with cold water, and cold 2N hydrochloric acid had suggested that the hydrazone was very sparingly soluble in these solvents. Quantitative tests, however, showed that the compound had a greater solubility than was allowable: under the experimental conditions which would be used for separating and washing the insoluble hydrazone, a 50 microgram sample was lost completely, while of 250 µg. and 500 µg. samples, only approximately 50% was recovered. This method was not investigated any further, since it was hoped to find some other method more sensitive than this.

2. Oxidation with periodic acid.

The unexpected failure to find a satisfactory method of estimating the acetaldehyde formed by the action of lead tetraacetate on pregnanetriol, suggested that it might be more profitable to estimate the 17-ketosteroid, which is the other product of fission. This approach has the advantage that the analytical method need not necessarily be so sensitive since, unlike acetaldehyde, the ketosteroid has practically the same molecular weight as pregnanetriol itself. Estimation of the steroid fission/

fission product entails one considerable disadvantage: the specificity of the method is no longer so great. Whereas only 17:20-dihydroxy steroids with a methyl group at C 21 can give rise to acetaldehyde, the structure at C 21 is immaterial, if the 17-keto-steroids formed are measured.

For this series of experiments, the reaction with lead tetraacetate was replaced by oxidation with periodic acid. This compound, as was discovered by Malaprade (1928, 1934), also causes quantitative oxidative fission of α-glycols. The reaction is less specific than that with lead tetraacetate, since oxidative fission of α-ketols and α-diketones occurs. It has the advantage over lead tetraacetate that it can be used in aqueous alcoholic solution. The efficacy of this reagent in oxidising steroids with hydroxyl groups at C 17 and C 20 has been demonstrated by Reichstein and others (Reichstein and Shoppee, 1943). Talbot and Eitingon (1944) have described a method of estimating such compounds in urine using periodic acid.

Two preliminary experiments were carried out on pregnanetriol, using potassium meta-periodate in the presence of sulphuric acid. After appropriate extraction/

extraction and washing, the 'formed' 17-ketosteroids were measured by means of the modification of Callow, Callow and Emmens (1938) of the Zimmermann (1935, 1936) reaction with m-dinitrobenzene, as follows:

To 0.2 ml. of the ethanolic solution to be estimated, 0.2 ml. of 2% (w/v) m-dinitrobenzene in ethanol, and 0.2 ml. of 2.50 N ethanolic KOH were added. The ethanol was aldehyde free in all cases; the dinitrobenzene was purified and tested as described by Callow et al. The reaction tubes were stoppered, and placed in a water bath at 25°C. ± 0.1° for one hour. At the end of this time the solutions were diluted with 10 ml. ethanol, and the colour read against a reagent blank in the Spekker photoelectric absorptiometer using Ilford spectrum green (604) filters.

The amounts of 17-ketosteroid formed are expressed in terms of mg. of androsterone, read from a calibration curve drawn up from the results of a large number of estimations on known amounts of androsterone. In addition, androsterone standards were run at the same time as the unknowns, to check the reliability of the reagents. Since aetiocholan-3α /

3α-ol-17-one, which is actually formed by oxidation of pregnanetriol, is more chromogenic than androsterone (Wilson and Nathanson, 1945), the yields appear to be slightly higher than was actually the case.

Oxidation with potassium metaperiodate.

Expt. 1. 0.445 mg. of pregnanetriol, dissolved in 2 ml. methanol, was allowed to react for one hour at 25°C. with 80 mg. $KIO_4$ dissolved in 4 ml. N $H_2SO_4$. The reaction mixture was transferred to a separating funnel with 100 ml. ether, and washed three times with N NaOH, shaking two minutes for each washing, and three times with 10 ml. $H_2O$ before evaporating to dryness. The dried residue was dissolved in 10 ml. ethanol, and four 2 ml. aliquots were taken for determination. (The aliquots were taken to dryness by heating in a water bath in a gentle stream of air; the residues were then dissolved in 0.2 ml. ethanol for the determinations).
Found: (average) 43 μg. 17-ketosteroids/estimation.
Calculated 77 μg. Recovery = 56% theoretical.

In/

In a second, otherwise exactly similar, experiment, the reaction was allowed to proceed for 17 hours. Forty-seven per cent. of the theoretical 17-ketosteroid content was found. It seems very probable that the concentration of potassium meta-periodate used was not sufficiently high. At this stage some periodic acid became available, and it was used in all the ensuing experiments.

Oxidation with periodic acid.

Expt. 2. 0.445 mg. pregnanetriol, dissolved in 2.25 ml. methanol, was allowed to react for one hour at 25°C. with 0.25 ml. of an aqueous solution containing 68 mg. periodic acid ($H_5IO_6$). The extraction and washing was carried out as in Expt. 1. Forty-five per cent. of the theoretical 17-ketosteroid content was found.

In this experiment the concentration of periodic acid was much the same as used by Talbot et al.(1944), but the addition of 0.04 c.c. of conc. sulphuric acid was omitted, and the low result appears to confirm Talbot's observation that the addition of sulphuric acid is essential if good recoveries are to be obtained.

Oxidation/

Oxidation with periodic acid in presence of sulphuric acid.

Expt. 3. 1.592 mg. pregnanetriol was dissolved in 3 ml. methanol and 0.25 ml. of water containing 68 mg. of periodic acid was added, followed by 0.05 ml. of concentrated sulphuric acid. The reaction was allowed to proceed for 75 minutes at 25°C.; the 17-ketosteroids, isolated as before, represented 85% of the theoretical yield.

Summary of further results

The reaction mixture consisted, in all cases, of 3 ml. methanol, 0.25 ml. water, 68 mg. periodic acid and 0.05 ml. conc. $H_2SO_4$. The reaction was allowed to proceed for one hour at 25°C., unless otherwise stated. Extraction, washing and estimations were carried out as previously described.

Table 1 /

Table 1.

Recovery of 17-ketosteroids from oxidation products of pregnanetriol.

| Wt. of triol mg. | 17-ketosteroids found. % theoretical | |
|---|---|---|
| 0.262 | 41 | |
| 0.524 | 72 | |
| 1.376 | 90 | Reaction allowed to proceed for 1½ hr. |
| 2.980 | 95 | |
| 3.680 | 96 | |
| 1.835 | 65 | Reaction allowed to proceed for 3 hr. |
| 2.005 | 94 | |
| 0.872 | 78 | |

It appears that under the experimental conditions used, amounts of pregnanetriol of 2 mg. or over can be estimated with an accuracy of over ninety per cent.

Since, unless a preliminary separation of the ketonic and non-ketonic fractions is carried out, the value obtained for pregnanetriol-like compounds in urine depends on the difference between 'pre-formed' or natural 17-ketosteroids, and 'total' 17-/

17-ketosteroids found after oxidation, it is essential that the chromogenic capacity of the naturally occurring 17-ketosteroids should not be affected by treatment with periodic acid. Androsterone was subjected to the oxidation reaction conditions for up to four hours. No appreciable change in the colour values obtained was observed. The slight loss is probably due to extraction, transference, and evaporation losses rather than to any effect of the periodic acid.

Table 2.

Recovery of added androsterone from periodic acid reaction mixture.

| Amount of androsterone added. mg. | Hours incubated with periodic acid. | Recovery % |
|---|---|---|
| 2.441 | 1 | 97 |
| 2.245 | 2 | 97 |
| 2.300 | 4¼ | 97 |

It was noticed on several occasions that, on evaporation, the ethereal extract of the oxidation product gave a brown, gummy residue. Talbot *et al.* also/

also observed this; they believed that the charring was due to residual oxidising agents, and that when charring took place, destruction of 17-ketosteroids occurred. In some cases they introduced washings with alkaline sodium hydrosulphite to prevent this happening; but they finally decided that this was not necessary provided the ether solution is shaken 'very extensively' with sodium hydroxide. It seemed possible that in some of the cases where the oxidation process appeared inefficient, the low recoveries might in reality be due to this factor. For this reason, when estimations were carried out on urine extracts, the number of alkaline and water washings was each raised to four. As before, each washing with sodium hydroxide was shaken for at least two minutes.

3. Estimation of pregnanetriol-like compounds in urine.

Butler and Marrian (1937) isolated pregnanetriol from urine which had not been subjected to any form of acid hydrolysis. Talbot et al. (1944) showed that the 17:20-dihydroxy compounds present in abnormal urines were acid labile; although Mason and Kepler (1945) /

(1945) isolated small amounts of pregnanetriol from urines which had been subjected to acid hydrolysis, it seems probable that such treatment causes considerable destruction of this type of compound. When Mason and Kepler hydrolysed the mixed glucuronide of pregnanediol and pregnanetriol which they isolated from one of their cases, they used an enzymic method.

Acid hydrolysis being suspect, the urine samples were collected without preservative, and incubated at 37°C. for one week before extraction. Marrian (1933) showed that by allowing bacterial growth to occur in urine, large amounts of oestrogenic material were 'freed'. The ability of micro-organisms to hydrolyse steroid conjugates has since been widely observed, and, more recently made use of, for example, by Cohen and Bates (1949, ~~1950~~), Brooksbank and Haslewood (1950) and Buehler, Katzman, Doisy and Doisy (1949).

(a) Estimation of pregnanetriol-like compounds in normal pregnancy urine.

After incubation at 37°C. without preservative, the urine was in each case brought to neutrality with hydrochloric acid, and extracted four times, with one/

one quarter its volume of peroxide-free ether. The combined ethereal extract was washed three times with one fourth its volume of N NaOH, three times with water, and taken to dryness. The residue was dissolved in ethanol, aliquots were taken for estimation of 'pre-formed' 17-ketosteroids, and an appropriate volume of the solution was taken to dryness, and subjected to periodic acid oxidation, as previously described. The oxidation product was dissolved in ethanol and aliquots taken for estimation of 'total' 17-ketosteroids.

Table 3.

'Pre-formed' and 'total' 17-ketosteroids excreted in normal pregnancy.

| Case | Hrs. incubated | 17-ketosteroids. Mg/l. | | |
|---|---|---|---|---|
| | | 'Pre-formed' | Total | Formed |
| NP 1 | 168 | 7.9 | 7.3 | |
| NP 1 | 336 | 9.9 | 10.4 | 0.5 |
| NP 2 | 185 | 6.5 | 7.5 | 1.0 |

The results shown in Table 3 indicate that a small amount of 'pregnanetriol-like' material is apparently excreted in normal pregnancy urine. In the urine NP 1 no 'formed' 17-ketosteroid was found after incubation for seven days, but 0.5 mg./l. was found after fourteen days' incubation. These amounts are considered to be minimal, since there is a tendency to lose non-specific chromogenic material during the periodic acid oxidation.

(b) Estimation of pregnanetriol-like compounds in the urine of four cases in which the adrenal cortex was believed to be hyperactive [x]

The urine, collected without preservative, was worked up in the same way as has been described for normal pregnancy urine, except that the incubation time was reduced, since the specimens were already several/

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x I am indebted to Dr A. Hain for making these urine specimens available to me.

several days old when they were received, and the weather was hot. The cases were as follows:

Case A1: woman, aged 21; pseudohermaphroditism.

Case A2: sister of A1, aged 17; do.

Case A3: girl, aged 2; diagnosed as having an adrenalcortical hyperplasia or tumour.

Case A4: woman with history of high 17-ketosteroid excretion - figures up to 100 mg./day.

The results obtained are shown in Table 4.

Table 4.

Pre-formed and total 17-ketosteroids excreted in abnormal urines.

| Case | Hrs. incubated | 17-ketosteroids - Mg./l. | | | Vol.of 24 hr. specimen ml. |
|---|---|---|---|---|---|
| | | Pre-formed | Total | Formed | |
| A1 | 72 | 8.0 | 13.3 | 5.3 | 1210 |
| A2 | 72 | 5.5 | 9.7 | 4.2 | 1160 |
| A3 | 72 | 31.5 | 73.7 | 42.2 | x |
| A4 | 144 | 13.8 | 14.5 | 0.7 | 1520 |

x 950 ml. of urine represented several days' collection.

All four cases show some excretion of pregnanetriol-like material. In one case (A3) the excretion of this material was strikingly high. Unfortunately no definite diagnosis of this case was available, nor was it possible to obtain further supplies of urine.

(c) Effect of progesterone administration on the excretion of 'preformed' and 'total' 17-ketosteroids.

During the course of an investigation of the metabolism of progesterone, fairly large doses of this compound were administered daily to certain subjects over a period of several weeks, and the daily excretion of pregnanediol was measured (Sommerville and Marrian, 1950). It was considered interesting to take this opportunity of estimating the 'preformed' and 'formed' 17-ketosteroids during the period of progesterone administration, and during a previous control period. In certain cases traces of toluene were unintentionally added to the bottles in which the 24-hour specimens were collected. Even a very small amount of toluene (less than 1 ml.) was sufficient to inhibit the bacterial hydrolysis of 17-ketosteroids to a considerable extent, although some/

some bacterial growth did occur in these specimens, as determined by change in pH. One of these results is included to demonstrate this effect.

Experimental.

The total volume of the 24-hour specimen, collected without preservative, was noted; in some cases the volume was made up to 2½ litres.

Acid Hydrolysis (in duplicate)

150 ml. of urine, or diluted urine, was adjusted to pH 1, and brought to the boil under reflux; 3 ml. conc. HCl (A.R.) was added, and the boiling was continued for 30 minutes. The urine was cooled, and extracted three times with 50 ml. of toluene; emulsions were broken by slow filtration through a Buchner filter funnel. The combined toluene ~~extract~~ extract was washed twice with 50 ml. N NaOH and twice with 50 ml. of water. The washed extract was transferred to a round-bottomed flask, and evaporated nearly to dryness on an electric hot-plate, and then taken completely to dryness under reduced pressure on a boiling water bath. The dry residue was dissolved in a known volume of ethanol, and suitable aliquots, usually 1/20th-1/25th, taken for 17-keto-steroid/

steroid estimation.

Bacterial hydrolysis.

A 400 ml. sample of the diluted urine (or, in some of the experiments, a sample of undiluted urine equivalent to one fifth of a 24-hour specimen) was incubated at 37°C. for 7-9 days. Concentrated hydrochloric acid was then added until the urine was approximately neutral. The urine was extracted three times with one third of its volume of peroxide-free ether, the combined ethereal extract was washed three times with N NaOH and three times with water, and taken to dryness. The residue was transferred to a 20 ml. volumetric flask with ethanol, and made up to the mark. Aliquots of this solution were taken for determination of 'preformed' 17-keto-steroids. Periodic acid oxidation was carried out, by the method previously described, on the residue from an aliquot of the bacterially-hydrolysed neutral fraction; usually an aliquot equivalent to one tenth of a 24-hour specimen was taken. All estimations were carried out, as usual, by the method of Callow, Callow and Emmens. The results are given in tabular form in the following pages (Tables 5-7).

Table 5.

Administration of progesterone to normal men.

| | 17-ketosteroids. Mg./24 hr. | | | Pregnanediol mg./24 hr. |
|---|---|---|---|---|
| | Acid[x] hydrol. | Bacterial hydrolysis | | |
| | | preformed | total | |
| **Subject M; receiving 40 mg. progesterone/day by mouth** | | | | |
| 5th day of administration | 18.5<br>22.3 | 14.8<br>15.1 | 15.1<br>15.5 | 4.38 |
| 18th day of administration | 19.6<br>17.9 | 13.5 | 12.1<br>11.7 | 4.65 |
| Control period | 15.4<br>15.6 | 27.0<br>27.0 | 24.7<br>23.8 | |
| **Subject P; receiving 60 mg. progesterone/day by mouth** | | | | |
| 8th day of administration | 17.4<br>16.2 | 11.4<br>11.5 | 13.8<br>13.0 | 11.53 |
| Control period | 20.5<br>20.3 | 24.2<br>24.0 | 23.2<br>22.6 | |

x The two figures for acid hydrolysed 17-ketosteroids represent duplicate estimations carried right through the hydrolysis and extraction. For bacterial hydrolysis, the two sets of figures are merely duplicate determinations on the same neutral fraction extracted.

Table 6.

Administration of progesterone to normal post-menopausal women,

| | 17-ketosteroids. Mg./24 hr. | | | Pregnanediol mg./24 hr. |
|---|---|---|---|---|
| | Acid hydrolysis | Bacterial hydrolysis | | |
| | | Preformed | Total | |
| Subject H[1] | | | | |
| Control period | 6.20 | 1.81[x] | 1.81[x] | |
| | 6.65 | 1.81 | 1.71 | |
| | 4.85 | 5.52 | 5.15 | |
| | 10.24 | 5.68 | 5.03 | |
| Administration period | | | | |
| 13th day | 4.36 | 4.47 | 3.39 | 13.1 |
| | 4.46 | 4.59 | 3.08 | |
| 14th day | 6.31 | 5.44 | 5.70 | 13.7 |
| | 5.42 | 5.44 | 5.65 | |
| Subject D[2] | | | | |
| Control period | 4.05 | 3.33 | 3.22 | |
| | 6.70 | 3.31 | 3.02 | |
| | 5.12 | 7.20 | 6.50 | |
| | 6.09 | 6.55 | 6.38 | |
| Administration period | | | | |
| 13th day | 5.87 | 5.71 | 5.99 | 12.8 |
| | 5.77 | 5.86 | 5.86 | |
| 14th day | 6.55 | 12.9 | 11.4 | 13.1 |
| | 6.81 | 12.1 | 11.2 | |

x Urine contaminated with traces of toluene.
1-Received 60 mg. progesterone/day by mouth.
2-Received 40 mg. progesterone/day by mouth.

Table 7.

Summary of results on effect of progesterone administration on excretion of 'preformed' and 'total' 17-ketosteroids.

| Subject | $\frac{\text{Total 17-ketosteroids}}{\text{Preformed 17-keto-steroids}} \times 100$ | | $\frac{\text{Bact.hydrol.17-ketost.}}{\text{Acid hydrol.17-ketost.}} \times 100$ | |
|---|---|---|---|---|
| | Control period | Administr. period | Control period | Administr. period |
| M | 90 | 102<br>88 | 174 | 73<br>72 |
| P | 95 | 117 | 118 | 68 |
| H | 91 | 71<br>104 | 74 | 103<br>93 |
| D | 94<br>94 | 103<br>90 | 62<br>122 | 99<br>188 |
| Average | 93 | 98 | 110 | 99 |

Discussion

No 'formed' 17-ketosteroid was detected in any of the control period specimens: the total 17-ketosteroids varied from 90 to 95 per cent. of the 'preformed' 17-ketosteroids, averaging 93 per cent. There was no consistent detection of 'formed' 17-ketosteroids during progesterone administration, but in four out of the seven estimations, a positive value was found. In only one case, however, was this outside the range of the experimental error of the colorimetric determination. On the other hand, the extra extractions and washings involved in the periodic acid oxidation appear to remove non-specific chromogenic material, so that there is a tendency for a loss of chromogenic capacity to occur; thus even a small increase in chromogenicity may be significant.

The results do show that there is no considerable excretion of pregnanetriol-like compounds, freed by bacterial hydrolysis, either normally, or during administration of progesterone. Further, that if small amounts of such compounds are excreted during progesterone administration, the excretion is/

is not constant, but varies considerably from day to day.

The results obtained do not exclude the possibility that small amounts of 17:20-dihydroxy-steroids are normally excreted, since a small increase in 17-ketosteroids following periodic acid oxidation may well be masked by the loss of non-specific chromogens. It should be noted, in this connection, that extracts of bacterially hydrolysed urine contain relatively small amounts of non-specific chromogens, compared with acid hydrolysed urine extracts.

This work was not proceeded with further, partly because it was felt that the method of hydrolysis used was not entirely satisfactory, and partly because of the difficulty experienced in obtaining urine specimens from suitable cases. When these obstacles are overcome, it is hoped to modify and extend the method used. The procedure employed by Talbot et al.(1944) of carrying out the periodic acid oxidation on the non-ketonic fraction of urine extracts would increase the specificity of the estimation, and also its sensitivity since most of the background reading due to preformed 17-keto-steroids/

steroids would be cut out. An alternative modification would be to estimate the acetaldehyde formed after oxidative fission with periodic acid, as had originally been intended in the lead tetraacetate reaction. This method would be specific for compounds possessing the same side chain as pregnane-3α;17α:20α-triol only if the method of estimating acetaldehyde was unaffected by the presence of formaldehyde.

Although, admittedly, the method of acid hydrolysis used (without simultaneous extraction) would be expected to cause some destruction of 17-ketosteroids, a comparison of the relative efficiency of acid and bacterial hydrolysis is nevertheless striking. The 17-ketosteroids found after bacterial hydrolysis ranged from 68% to 188% (average 104%). of the corresponding values after acid hydrolysis. The occasion on which bacterial hydrolysis liberated 188% of the ketosteroids liberated by acid hydrolysis is a surprising testimonial both to the efficacy of bacterial hydrolysis, and to the destructiveness of acid hydrolysis, unless bacterial synthesis of 17-ketosteroids is supposed to have occurred.

Summary /

Summary

1. Unsuccessful attempts have been made to estimate pregnane-3α:17α:20α-triol by means of the acetaldehyde formed by the oxidative fission of the triol with lead tetraacetate.

2. The reaction of periodic acid with pregnane-3α:17α:20α-triol has been shown to give over 90% of the theoretical yield of 17-ketosteroids, under the conditions used.

3. Making use of the hydrolytic powers of the bacteria which occur naturally in unpreserved urine, the urine of two normal pregnancy cases and four cases of suspected adrenalcortical hyperactivity have been examined for pregnanetriol-like compounds (i.e. compounds giving rise to 17-ketosteroids on periodate oxidation). A very small amount of pregnanetriol-like material was found in the pregnancy urine, and in one of the abnormal cases, and rather more, 4.9 and 6.4 mg. per 24 hr., in two of the other abnormal cases. In the fourth case the 'pregnanetriol' excretion was estimated to be 42 mg. per litre.

Using similar methods of hydrolysis and extraction, none of the normal cases gave any apparent pregnanetriol/

pregnanetriol.

Results obtained during the administration of large doses of progesterone to two normal men and two normal post-menopausal women show that no appreciable proportion of the progesterone administered is converted into 'pregnanetriol-like' compounds. There is some indication that there may be a slight variable formation of pregnanetriol-like compounds under these circumstances, but it is felt that the method of hydrolysis available is too open to criticism to make such slightly positive results valid.

## SECTION III.

## SEPARATION OF THE NON-KETONIC SODIUM GLUCURONIDATE FROM THE 'SODIUM PREGNANEDIOL GLUCURONIDATE' ISOLATED FROM HUMAN PREGNANCY URINE BY THE METHOD OF VENNING.

A. 1. A general introduction to the urinary conjugated steroids.

2. 'Sodium pregnanediol glucuronidate' isolated by the method of Venning.

B. Experimental.

1. Preparation of starting material.

2. The estimation of 20-ketosteroids and of pregnanediol.

3. Various unsuccessful attempts to prepare pure sodium pregnanediol glucuronidate.

4. The purification of the non-ketonic glucuronidic acid by means of trimethylammoniumacetohydrazide chloride:

Results
Experimental

C. Summary.

1. A general introduction to the urinary conjugated steroids.

The discovery of the presence of the steroid hormones and their metabolites in urine was due to the observation of the activity of the urine in various biological sex hormone tests. It was early observed that this biologically active material could be extracted from the urine by means of immiscible fat solvents. Later, it was found that preliminary acidification of the urine noticeably increased the amount of active material which could be extracted (Doisy, Veler and Thayer, 1930; Marrian, 1930).

In the following years it gradually came to be realized that all of the early work had only been dealing with a small fraction of the total excreted steroids. This situation was first made clear with respect to the oestrogens. In 1933, Marrian reported that a very large increase in the oestrogenic activity extractable from human pregnancy urine occurred after boiling with acid. Later/

Later, systematic investigations of the hydrolysis and extraction conditions required to give maximum yields of oestrogens by Borchardt, Dingemanse and Laqueur (1934), and Cohen and Marrian (1935) confirmed this finding. Once the existence of water-soluble, acid-hydrolysable forms of the oestrogenic hormones was admitted, attention was turned to the problem of isolating these compounds.

In 1936, Cohen and Marrian were able to report the isolation of an oestriol complex from human pregnancy urine; its analyses and properties showed it to be oestriol monoglucuronide. No conjugated form of oestrone, nor of oestradiol, has yet been isolated from human urine, but Schachter and Marrian (1938) have isolated the sulphate of oestrone from the urine of pregnant mares. According to Butenandt and Hofstetter (1939), the behaviour of the oestrone conjugate fraction of human pregnancy urine suggests that it too is probably the sulphate. Odell and Marrian (1936) reported that pregnanediol was also present in human pregnancy urine in an acid-hydrolysable/

hydrolysable form. Like oestriol, it proved to be excreted as the glucuronide, sodium pregnanediol glucuronidate being isolated by Venning and Browne later in 1936.

The situation with regard to the urinary androgens developed in a similar manner, at a somewhat later date. Although Addler (1934) reported that the amount of androgenic material extractable from male urine could be considerably increased by boiling with acid, Gallagher, Koch and Dorfman (1935) were unable to confirm this finding. It was therefore proposed by them that androgens were not excreted in a conjugated form. Later, Peterson, Gallagher and Koch (1937) found that brief acid hydrolysis did cause an increase in androgenic activity, their previous failure to observe this being attributed to the loss of biological activity which occurs on prolonged boiling with acid.

In the following year, Peterson, Hoskins, Coffman and Koch (1938) reported that all the androgenic activity in acidified, unboiled, fresh male urine could be extracted with butanol, and that such/

such extracts contained practically all the androgens in a conjugated, inactive form, the activity being liberated by mild acid hydrolysis. McCullagh and Osborn (1938) obtained similar results. It thus became accepted that the androgens, like the oestrogens and pregnanediol, are excreted as acid-hydrolysable, water-soluble conjugates. It seems probable that much of the early confusion on the subject was caused by working with urine specimens in which some bacterial hydrolysis had already occurred.

Peterson et al. (1938) considered that the androgen conjugates probably consisted of androsterone and dehydroisoandrosterone hexuronides. This belief has not yet been confirmed by the isolation of these compounds, the androgen complexes so far isolated both being sulphates: dehydroepiandrosterone sulphate from normal male urine by Munson, Gallagher and Koch (1944), and androsterone sulphate from the urine of a man suffering from an interstitial cell tumour of the testis, by Venning, Hoffman and Browne (1942).

The/

The site of formation of the steroid conjugates.

Although there is very little direct evidence on the subject, the liver is generally presumed to be the principal site of synthesis of the steroid conjugates. Some experimental support of this is afforded by the fact that advanced cases of cirrhosis of the liver have been reported to excrete oestrogens in the free state (Glass, Edmondson and Soll, 1940); but the belief is mainly founded on work which has been done on the 'detoxication' of such compounds as phenol. Glucuronide formation from these compounds has been shown to occur almost exclusively in the liver (Hemingway, Pryde and Williams, 1934; Lipschitz and Bueding, 1939); the kidney was the only other tissue which Lipschitz et al. found to be active in this respect. The observation of De Meio and Arnolt (1944) that intestine and spleen were also active in the formation of 'combined phenol' suggests that some sulphate formation may occur in these tissues.

While it has been amply demonstrated (e.g. by Biskind et al.,1941-43) that, in vivo, the liver is the main site of inactivation of the oestrogens, and of testosterone, Samuels, McCaulay and Sellers (1947)/

(1947) were unable to find any evidence that conjugation was a major factor in the inactivation of testosterone by liver tissue, in vitro. Heller (1940) and De Meio, Rakoff, Cantarow and Paschkis (1948) reported similar results concerning the inactivation of oestradiol by liver tissue; but these results for oestradiol both depend on biological methods of assay involving relatively large errors. On the other hand, Crepy (1946) has claimed that oestrone, oestradiol and oestriol are all conjugated as glucuronides by liver slices; the chemical estimations of the combined oestrogens and combined glucuronic acid agreed well for oestradiol and oestriol, but there was a discrepancy in the figures for oestrone, suggesting that it was also conjugated other than as the glucuronide, possibly as the sulphate. Clark, Kochakian and Lobotsky (1947) found that after incubating $\Delta^4$-androstenedione-3,17 with liver slices, a water-soluble compound, whose properties suggested that it resembled dehydroisoandrosterone glucuronide, was formed.

While there is very little satisfactory evidence to show that the liver is in fact the main site/

site of formation of the steroid conjugates, this assumption does not appear unreasonable on general grounds, and in the absence of any direct evidence to the contrary.

Methods which have been used in the isolation of urinary conjugated steroids.

Extraction of the fresh urine with butanol, with or without a previous adjustment of pH, has been the first step in all the isolation procedures described. The subsequent steps have usually been chosen by trial and error, the various fractions being tested chemically or biologically after each step of the attempted purification, and the concentration and recovery obtained of the desired compound thus determined.

In the isolation of oestriol glucuronide (Cohen et al., 1936) it was possible to make good use of its phenolic nature, the extracted material being repeatedly partitioned between aqueous alkali and immiscible organic solvents, or water and immiscible organic bases. Partitioning between two solvents was also one of the main features in the method used by Munson et al. for isolating dehydroepiandrosterone sulphate; the neutral butanolic extract/

extract was extracted exhaustively with water; the fractions least soluble in water contained the dehydro-~~iso~~epiandrosterone sulphate.

A fractionation depending on the solubility, or insolubility, of the complex in a definite concentration of aqueous acetone was used by Venning and her coworkers in the isolation of both sodium pregnanediol glucuronidate and androsterone sulphate (Venning, 1936; Venning and Browne et al., 1942). Klyne, Schachter and Marrian (1948) used a similar method in the isolation of $\Delta^{16}$-allopregnen-3β-ol-20-one sulphate from pregnant mares' urine.

The inorganic and organic salts of the steroid conjugates have been used in widely different ways in the various isolation procedures. Bisset, Brooksbank and Haslewood (1948) concentrated their 'pregnanediol-like glucuronide', consisting chiefly of the glucuronide of $\Delta^{16}$-androsten-3α-ol (Brooksbank and Haslewood, 1950), by entrainment of the barium salts on a precipitate of barium phosphate, while barium salts were also made use of in the final stages of the purification of oestriol glucuronide and oestrone sulphate. The sulphate conjugates/

conjugates are often unstable in the free acid form; although they may be prepared as their alkali metal salts, as was oestrone sulphate (Schachter et al., 1938), these are often difficult to separate from inorganic impurities, and do not always melt sharply. Klyne and his coworkers (Klyne and Marrian, 1945; Klyne, 1946; Klyne, Schachter and Marrian, 1948; Paterson and Klyne, 1948) have overcome these difficulties by isolating the sulphate conjugates of pregnant mares' urine by means of the salts formed with p-toluidine/piperazine hydrochloride.

Finally, some of the steroid conjugates are sufficiently soluble in non-aqueous solvents to permit them to be separated by chromatography on an alumina column. Androsterone sulphate was purified in this manner, acetone containing increasing concentrations of ethanol being used to develop the chromograph, while Brooksbank et al. (1950) used butanol-methanol, pure methanol, and methanol-acetic acid to elute different fractions of the 'pregnanediol-like glucuronide'.

This introduction has not been intended to cover all the aspects of the urinary conjugated steroids/

steroids, or to give a comprehensive review of the literature on this subject, but merely to provide a general background to the work which is described in the following two sections.

## 2. 'Sodium pregnanediol glucuronidate' isolated by the method of Venning.

Of the various methods of isolating steroid conjugates which have been briefly referred to, that of Venning and Browne (1936) for the isolation of sodium pregnanediol glucuronidate[x] is probably the simplest, and also the most important, since it forms the basis of a widely used method for estimating the urinary excretion of pregnanediol (Venning, 1937, 1938).

While this method is probably reasonably accurate/

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x The term 'NaPG' is hereafter used to refer to the material obtained in the sodium pregnanediol glucuronidate fraction from normal pregnancy urine using the Venning method.

accurate for urine specimens containing 10-15 mg. of pregnanediol per 24 hr. or more, many workers have found it unreliable at low levels of pregnanediol excretion (Astwood and Jones, 1941; Sommerville, Gough and Marrian, 1948). Moreover, the findings of Mason and Kepler (1945) on pregnanetriol glucuronide, and of Marrian and Gough (1946) on pregnanolone glucuronide, show that not only is the Venning method not specific for pregnanediol glucuronide, but that it is often impossible to purify the 'NaPG' from contaminating glucuronides by repeated recrystallization. Results described later in this and the following section, show that the melting point of 'NaPG' is not a good criterion of purity when the contaminant is a closely related glucuronide.

An investigation of the purity of the 'NaPG' isolated from human pregnancy urine was suggested to Marrian et al. (1946) by the consistently low results which they and other workers (Astwood et al., 1941; Talbot, Berman, MacLachlan and Wolfe, 1941) obtained when estimating the recovery of free pregnanediol after acid hydrolysis of 'NaPG'. They were able to show that the 'NaPG' gave rise to only approximately 80%/

80% of the theoretical amount of pregnanediol, pregnan-3α-ol-20-one accounting for probably most of the other 20%. The presence of pregnanolone was not due to the hydrolysis conditions, since the unhydrolysed 'NaPG' gave a brick-red colour in the Zimmermann reaction. Nor was it due to bacterial action in the urine, since 'NaPG' samples prepared from urine collected under sterile conditions, gave the same colour as 'NaPG' prepared from normally collected urine.

Marrian et al. (1946) were unable to separate pure sodium pregnanediol glucuronidate from its ketonic contaminant by repeated precipitation from aqueous acetone with dry acetone or by repeated recrystallization from 90% aqueous ethanol. Kyle (1950) has recently shown that 'NaPG' is also contaminated with sodium allopregnane-3α:20α-diol glucuronidate, but that this compound can be removed by repeated acetone precipitation.

The observation of the impurity of 'NaPG' was of considerable importance, since it then became apparent that methods which had been developed for estimating urinary pregnanediol as the/

the free compound, were much more efficient than had previously been supposed. Since the methods described for estimating free pregnanediol are less laborious and time-consuming than the Venning method, as well as being more accurate and sensitive (Sommerville et al., 1948), they are now very widely used.

The work described in this and the following section was suggested by the observations of Marrian and Gough. It was decided to attempt the separation and characterization of the pure sodium pregnanediol glucuronidate, and, if possible, to isolate for the first time the sodium pregnanolone glucuronidate which was presumed to be the other principal component of the mixture. Since the 'NaPG' isolated by the method of Venning consists very largely of these two fractions, it was used as starting material for all the separation procedures described.

B. Experimental.

1. Preparation of starting material

Daily collections of human pregnancy urine were made at the Simpson Memorial Maternity Pavilion. The urine was in all cases preserved with butanol, 200 ml. being added to each winchester.

The 'NaPG' used as starting material in these experiments was prepared in one of the following ways:-

Method I. Step 1. The urine was adjusted to pH 2.0[x] (Lovibond comparator, thymol blue indicator) with concentrated hydrochloric acid, and then extracted twice with one third its volume of butanol. Emulsions were separated by centrifugation. The combined/

x The object of this adjustment of pH was to increase the efficiency of the extraction of oestriol glucuronide, which was prepared from the same butanol extracts.

combined butanol extract was washed once with one fifth its volume of water, three times with one fifth its volume of N/3 NaOH, and finally three times with a similar volume of water. The washed extract was distilled almost to dryness at reduced pressure, the temperature being kept below 80°C. The residue was transferred to a small flask with 80% aqueous ethanol and stored in the refrigerator. The residues from other extracts were added until a suitable amount of material for working up had accumulated.

Step 2. The combined residues were then transferred to a 5 l. round-bottomed flask, and the solvents removed at the pump, until frothing made this impossible. The residue was transferred to a large separating funnel with N NaOH and extracted four times with butanol. The combined butanol extract was washed four times with one fourth its volume of water, both phases being allowed to clear completely at each washing. The butanol was distilled off at reduced pressure.

Step 3. Aqueous acetone (50%) was added, with warming, to the residue until no more would dissolve. The solution was cooled and filtered. Sufficient

dry/

dry acetone was added to the filtrate to make the final acetone concentration 95%. After standing overnight in the refrigerator, the supernatant acetone was carefully decanted through the filter, and the 'NaPG' filtered off, washed with dry acetone, and dried in a vacuum desiccator over calcium chloride.

Method Ia.

The same as Method I, except that the crude butanolic extracts were stored in butanol instead of in 80% ethanol.

Method II.

The same as Method I, except that after the initial water washing of the butanol extract, 500 ml. of butanol containing 2 g. of dissolved NaOH were added to the butanol extract. It was hoped in this way to reduce the number of alkali washings needed, but in practice it was found to be difficult to make the extract alkaline by this method, and it was therefore abandoned.

Method III.

The urine was adjusted to pH 2.0 with conc. HCl and extracted three times with one third its volume/

volume of butanol. The combined butanol extract was washed as follows:- once with 1/10 volume of water, once with 1/6 volume of N/10 NaOH, once with 1/10 volume of N/10 NaOH, and finally with 1/10 volume of water. In Step 2, the butanolic extract was allowed to stand until clear, and then washed twice with 1/10 volume of water, before taking to dryness.

Method IV.

The urine was adjusted to pH 3.5 with conc. HCl (indicator, bromophenol blue), and extracted three times with 1/3 volume of butanol. The combined butanol extract was allowed to stand several hours, and any aqueous phase was run off; it was then washed three times with 1/10 volume of N/3 sodium hydroxide, and once with the same amount of water. Step 2 was carried out as in Method III, and Step 3 as described in Method I.

The yields obtained in the various preparations are shown in Table 8.

Table 8/

Table 8.

'NaPG' isolated from pooled late pregnancy urine by different methods.

| No.of days collection | Total vol. of urine extracted litres | Wt.of 'NaPG' isolated. g. | Yield of 'NaPG' mg./l. | Method of isolation used |
|---|---|---|---|---|
| 24 | 174 | 2.06 | 11.8 | I |
| 22 | 150 | 1.58 | 10.5 | I |
| 14 | 80 | 0.63 | 7.9 | Ia |
| 18 | 90 | 2.40 | 19.2 | Ia |
| 10 | 35 | | | II |
| 17 | - | 4.15 | - | III |
| - | 130 | 5.32 | 35.5 | IV |
| - | 265 | 14.5 | 54.7 | IV |

Methods III and IV gave very much better yields than the earlier methods. A small amount of this apparent gain may be due to inorganic impurities, since the water-washings were cut to a minimum to prevent losses.

It seems possible that higher yields of a purer product would be obtained if each extract were processed separately right through to the acetone precipitation/

precipitation stage (Step 3). Experience suggests that some destruction of 'NaPG' occurs during the storage of the crude extract.

2. The estimation of 20-ketosteroids and of pregnanediol.

The 20-ketosteroid estimations referred to in this section were carried out by the modification of Callow, Callow and Emmens (1938) of the Zimmermann (1935, 1936) reaction with m-dinitrobenzene. The details of this method have already been given in Section II (p. 29).

In the case of glucuronide fractions, approximately 5 mg. of the material was tested, suspended in 0.2 ml. ethanol, instead of the usual 0.2 ml. of ethanolic solution; after the colour had been developed the pink colour of the turbid solution was assessed as 0 or varying from + to ++++. When estimations were carried out on free steroid fractions, aliquots which were expected to contain between 0.1 and 0.5 mg. of pregnanolone were taken for estimation. The colours developed were/

were read against ethanol in the Spekker photoelectric absorptiometer, using Ilford spectrum blue-green (603) filters. All estimations were carried out in duplicate, and the readings corrected for the reagent blank, which was run in duplicate at the same time. The pregnanolone value of the corrected readings was read off a standard curve, constructed with each set of reagents using known amounts of pure pregnan-3α-ol-20-one.

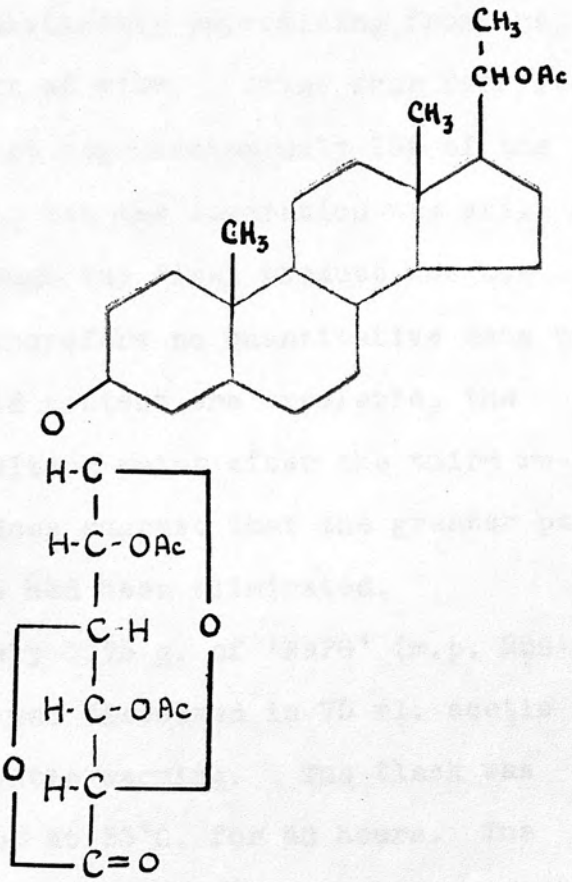
The pregnanediol content of free steroid fractions was estimated according to the colorimetric method of Talbot, Berman, MacLachlan and Wolfe (1941). The yellow colour developed in the presence of concentrated sulphuric acid was measured in the Spekker photoelectric absorptiometer using Ilford spectrum violet (601) filters, and the pregnanediol value read off a standard curve constructed using known amounts of pure pregnane-3α:20α-diol. Pregnan-3α-ol-20-one gives practically no colour in this reaction; its chromogenicity is approximately one-thirtieth of that of pregnanediol.

3. /

3. Various unsuccessful attempts to prepare pure sodium pregnanediol glucuronidate.

(a) By recrystallization of acetylated 'NaPG'

On acetylation 'NaPG' yields a non-acidic polyacetate, the analyses and properties of which indicate a triacetate lactone. Heard, Hoffman and Mack (1944) have shown that it has the following structure:-



It has already been shown (Marrian et al., 1946) that repeated recrystallization of 'NaPG' itself does not result in the elimination of the ketonic impurity, but it seemed possible that recrystallization of the acetylated derivatives might be more successful.

While some separation was achieved by this method, since the concentration of material chromogenic in the Zimmermann reaction was definitely reduced, it was distinctly unpromising from the quantitative point of view. After four recrystallizations, the product represented only 15% of the starting material, yet the separation was still not complete. Although the final product was not hydrolysed, and therefore no quantitative data on its 20-ketosteroid content are available, the sudden rise in melting point after the third recrystallization does suggest that the greater part of the impurities had been eliminated.

Approximately 0.75 g. of 'NaPG' (m.p. 269-270°, uncorr. decomp.) was dissolved in 75 ml. acetic anhydride, with gentle warming. The flask was stoppered and kept at 33°C. for 48 hours. The reaction mixture was poured into 500 ml. cold water contained/

contained in a separating funnel; a white crystalline precipitate formed. The flask was rinsed out with a further 175 ml. water, and the combined aqueous solution was extracted three times with 250 ml. of ether. The ethereal extracts were combined, washed three times with 250 ml. water, and taken to dryness. Traces of acetic acid were removed by the addition, and subsequent removal at reduced pressure, of several small volumes of ethanol. Yield: 0.78 g. After crystallization from absolute ethanol the product melted slowly over the range 95-110°. The once, twice and thrice recrystallized products melted at 117-120°, 116-121° and 144-145° respectively. After four recrystallizations from absolute ethanol, 0.127 g. of crystals, melting sharply at 146-147° were obtained. (All melting points are uncorrected). Heard, Hoffman and Mack (1944) gave the melting point of their twice recrystallized acetate as 123-125°.

Using 6.4 and 6.2 mg. of the four times recrystallized acetylated 'NaPG', weak positive reactions for 20-ketosteroids were obtained.

(b ). /

(b). Chromatography of the acetylated derivatives.

The much greater solubility of the acetylated 'NaPG' in non-aqueous solvents raised the possibility of separating the non-ketonic and ketonic fractions by chromatographing the mixed acetates on an alumina column. The attempt was given up when benzene-ethanol (3:1) and benzene-pyridine (19:1) failed to elute the compounds. Possibly much higher concentrations of polar solvents should have been tried. Brooksbank et al. (1950) used butanol-methanol, and methanol-acetic acid mixtures to elute fractions of their pregnanediol-like glucuronide from an alumina column.

(c) Partition chromatography of the free acid on a starch column.

Synge (1944) has described a method in which partition chromatography on a starch column was used for the separation of the partial hydrolysis products of gramicidin. The same procedure was applied to the acid derived from 'NaPG', but without securing any separation of the two fractions. The rate of elution rose steadily to a single peak, and then/

then declined gradually at a steady rate. Seven of the sixteen fractions collected were tested for 20-ketosteroids; all gave an apparently identical reaction.

Approximately one gram of 'NaPG' was dissolved, with slight warming, in 300 ml. of butanol which had previously been saturated with water; 2 ml. glacial acetic acid were added, and the butanol was then washed three times with 100 ml. portions of water. The washed butanolic solution was distilled to dryness at reduced pressure, and the residue dried at room temperature, in vacuo, over $CaCl_2$ and KOH.

The column was packed with starch suspended in water-saturated butanol. The impure pregnanediol glucuronic acid, 0.995 g., was dissolved in 20 ml. of water-saturated butanol and poured on to the column. Water-saturated butanol was also used for developing the chromatograph; the fractions eluted are recorded in Table 9.

Table 9 /

Table 9

| No.of fraction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Volume,ml. | 20 | 20 | 10 | 10 | 10 | 10 | 10 | 10 |
| Weight,mg. | 2.8 | 1.5 | 1.0 | 0.6 | 6.1 | 162.7 | 215.7 | 172.4 |

| No.of fraction | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|
| Volume,ml. | 10 | | | | | | | |
| Weight,mg. | 114.4 | 86.7 | 61.1 | 43.5 | 31.0 | 19.3 | 13.7 | 12.3 |

Fractions 6, 7, 8, 9, 10, 12 and 14 were tested for 20-ketosteroids. To obtain a representative sample, each fraction was dissolved in the calculated amount of ethanol to give a solution containing 10 mg./ml., and 0.5 ml. aliquots were taken for estimation. All gave a uniformly pink, slightly turbid solution on dilution of the reaction mixture.

(d) Formation of the semicarbazone.

Munson et al. (1944) separated dehydro~~iso~~epi-androsterone sulphate as its insoluble semicarbazone from aqueous solution. An experiment carried out to see whether 'NaPG', under similar conditions, would give rise to a precipitate of the semicarbazone of pregnanolone glucuronide, was unsuccessful; either the semicarbazone was not formed under the conditions used, or the volume of water was sufficient to keep it in solution. In a second experiment, the 'NaPG' was dissolved in a much smaller volume of aqueous alcohol, but still no precipitate was formed.

Experiment 1. 0.20 g. of crude 'NaPG' was dissolved in 200 ml. water and 0.50 g. semicarbazide hydrochloride and 0.64 g. sodium acetate ($CH_3.COONa.3H_2O$) were added. The reaction mixture was allowed to stand four days at room temperature; no precipitate was formed, nor did one form on subsequent heating for one hour on a boiling water bath.

Experiment 2. 0.20 g. 'NaPG' was dissolved in 20 ml. 50% aqueous ethanol; 0.1 g. semicarbazide hydrochloride and 0.15 g. sodium acetate were added, dissolved in the minimum volume of water. After standing/

standing five days at room temperature, no crystalline precipitate had formed.

(e) Attempted separation by means of the silver salt of the thiosemicarbazone of pregnanolone glucuronide.

Thiosemicarbazide reacts with aldehydes and ketones to yield thiosemicarbazones which form sparingly soluble silver salts. It was planned to use this reaction to purify 'NaPG'; after reaction with thiosemicarbazide, the addition of silver nitrate should precipitate the thiosemicarbazone, along with the excess thiosemicarbazide. The non-ketonic fraction should be readily extractable from the soluble material, while treatment of the precipitate with mineral acid should regenerate the ketonic fraction.

No success was achieved with this method; the fact that the 20-ketosteroid content of the non-ketonic fraction obtained was apparently no different from that of untreated 'NaPG' suggests that the thiosemicarbazone was never formed. Although a preliminary experiment showed that 'NaPG' was not precipitated from 70% ethanol by the addition of 10% $AgNO_3$, from the very low recovery obtained it appears that/

that entrainment of the silver salt of pregnanediol glucuronide on the precipitate of silver thiosemicarbazide may have occurred.

0.23 G. 'NaPG' was dissolved in 20 ml. 70% ethanol; approximately 0.1 g. of thiosemicarbazide was added, along with a further 5 ml. of 70% ethanol which was found necessary to dissolve it. The reaction mixture was allowed to stand 72 hr. at room temperature; it was then transferred to a centrifuge tube and 10% $AgNO_3$, in 70% ethanol, added until no further precipitation occurred. The precipitate was separated by centrifugation, the supernatant removed by suction and saturated with $H_2S$. The precipitate of $Ag_2S$ was filtered off, washed with aqueous ethanol, and the washings were added to the filtrate, which was then evaporated to a small volume under reduced pressure. This step removed the $H_2S$ and most of the ethanol. The remaining solution was transferred to a separating funnel with N NaOH and extracted with butanol; the butanol extract was washed with water and distilled to dryness under reduced pressure. The yield was only 0.082 g., or approximately 25% of the starting material.

The total product was dissolved in 10 ml. ethanol/

ethanol and two 0.5 ml. aliquots ( ≡ 4.1 mg.) were taken for 20-ketosteroid estimation. The colour produced was almost indistinguishable from that produced by 5.5 mg. untreated 'NaPG'.

(f) Girard separation of acetylated 'NaPG'

The most commonly used method of separating ketonic from non-ketonic fractions in steroid chemistry is probably by means of one of the reagents described by Girard and Sandulesco (1936), trimethylammoniumacetohydrazide chloride.

$$(CH_3)_3 \equiv \overset{+}{N} - CH_2 - CO - NH - NH_2 \quad Cl^-$$

This compound, reagent T, combines with ketones to give water-soluble hydrazone derivatives; non-ketonic substances may then be separated by extraction with an immiscible non-hydroxylated solvent. The difficulty in applying this method to the separation of sodium pregnanediol glucuronidate from its ketonic contaminant, lies in the very low solubility of the conjugate in ether and other possible extraction solvents.

Because/

Because of its much greater solubility in ether, it was felt that acetylated 'NaPG' might prove a more suitable starting material for such a separation than 'NaPG' itself. Two experiments were carried out to test this possibility. In the first of these the Girard derivative was prepared in an ethanolic solution of acetic acid, as described by Girard et al. (1936). The purification of the non-ketonic fraction obtained was very satisfactory, but large losses of non-ketonic material occurred, apparently due to deacetylation, and the accompanying loss of ether-solubility.

In an attempt to avoid this happening, the Girard derivative in the second experiment was prepared by short heating in glacial acetic acid according to the method of Talbot, Butler, MacLachlan and Jones (1940). Far from improving the situation, this technique seems to have caused considerable destruction of the glucuronide linkage. Further, the lack of any purification of the non-ketonic fraction suggests that the Girard derivative had not been satisfactorily formed under these conditions/

conditions.

Experiment 1. 0.59 g. crude acetylated 'NaPG' was refluxed for 1 hr. in 10 ml. absolute ethanol containing 0.75 ml. glacial acetic acid and 0.5 g. Girard's reagent T. The reaction mixture was cooled, and poured into a beaker containing 12 ml. N NaOH, 38 ml. water and 50 g. ice and mixed well. The mixture was transferred to a separating funnel and extracted three times with 50 ml. ether (non-ketonic fraction). Conc. HCl was added to the aqueous phase to make it approximately 0.5 N, it was allowed to stand for 1 hr. at room temperature, and was then re-extracted three times with 50 ml. ether (ketonic fraction).

Both ethereal extracts were washed once with 50 ml. 5% $NaHCO_3$ and three times with 20 ml. water. On distilling off the ether, and drying the residues, a non-ketonic fraction of 0.118 g. and a ketonic fraction of 0.037 g. were obtained.

Thus only approximately 25% of the starting material had been recovered. To determine where the loss had occurred, the aqueous washings and the final/

final aqueous solution were extracted with butanol, and the butanol was washed and taken to dryness. The washings of the non-ketonic and ketonic extracts yielded 25 mg. and 73 mg. respectively, but 337 mg. was extracted from the final aqueous solution, showing that the main amount of material lost had never been ether extracted.

The non-ketonic and ketonic fractions were saponified by refluxing for 30 min. in 5% (w/v) methanolic sodium hydroxide solution, and the glucuronide fractions were obtained by extraction with butanol, after most of the methanol had been distilled off under reduced pressure. The washed butanol extracts were taken to dryness and the residues recrystallized from 90% aqueous ethanol.

The non-ketonic fraction melted at 267-268° (uncorr., with decomp. and evolution of gas); 5.6 mg. gave an apparently negative Zimmermann reaction. The ketonic fraction melted at 245-250° (uncorr., decomp. and evolution of gas); 3.1 mg. of this fraction gave a considerably stronger colour in the Zimmermann reaction than 5.8 mg. of untreated 'NaPG'.

Experiment/

Experiment 2. 0.606 g. 'NaPG' triacetate was dissolved in 5 ml. glacial acetic acid, approximately 0.2 g. of Girard reagent T was added, and the mixture heated in a boiling water bath, under an air condenser, for 10 min. The mixture changed to a deep red colour; it was cooled under the tap, and then poured into 125 ml. of ice-cold water containing sufficient NaOH to neutralize $9/10^{th}$ of the acetic acid. The mixture was transferred to a separating funnel, and extracted four times with 50 ml. of ether (non-ketonic fraction).

The ether-extracted aqueous phase was acidified with 7 ml. conc. HCl and allowed to stand at room temperature for 1 hr. The ether extraction was then repeated (ketonic fraction).

Both ether extracts were washed with 5% $NaHCO_3$ and water and evaporated to dryness. The non-ketonic fraction was a yellow semicrystalline solid and weighed 0.360 g. The ketonic fraction weighed only 0.010 g.

Butanol extraction of the various aqueous fractions was again used to locate the losses; 6 mg. was recovered from the non-ketonic washings, 12 mg. from/

from the ketonic fraction washings and 61 mg. from the residual aqueous solution.

After saponification, the non-ketonic fraction yielded a product which melted slowly over the range 215° to 232°. Precipitation with dry acetone from aqueous acetone solution gave 12 mg. of good quality 'NaPG', which gave a normal positive test in the Zimmermann reaction. A considerable amount of the remaining non-ketonic fraction appeared to consist of free pregnanediol, judged by its solubility behaviour and melting point.

4. The purification of the non-ketonic glucuronidic acid by means of trimethylammonium-acetohydrazide chloride.

Results:

Since it is possible to extract with ether the glucuronidic acids from an acidified solution of their sodium salts, it should be possible to separate the ketonic and non-ketonic acids using Girard's reagent T. A preliminary experiment in which the reagent T was omitted showed that the glucuronide linkage in 'NaPG' was stable under the conditions/

conditions for forming the hydrazone used by Girard et al. (1936). However, it had previously been observed that it was impossible to extract pregnanediol glucuronidic acid with ether after nine-tenths of the acetic acid of the reaction mixture had been neutralized: presumably the pregnanediol glucuronidic acid is largely ionized at the pH attained. Accordingly the possibility of effecting the separation, omitting the partial neutralization of the acetic acid, was investigated.

It was realized that this procedure was bound to be inefficient in view of the low ether solubility of pregnanediol glucuronidic acid, and the slow hydrolysis of the ketonic complex which would occur in the acid solution while the non-ketonic fraction was being extracted, but it was hoped that by repeating the separation several times, a pure product might be obtained.

Working as rapidly as possible and with all the solutions and apparatus chilled in order to minimize the hydrolysis of the ketonic complex, a 'non-ketonic' fraction was obtained, which yielded after two further successive treatments in the same/

same manner, a final product which gave a virtually negative Zimmermann reaction. After purification and drying at 137°, a sodium salt, analysing in good agreement with the formula $C_{27}H_{43}O_8Na$, was obtained (m.p. 283.5-284.5° corr., decomposition and evolution of gas). This gave a strongly positive Tollens reaction for glucuronic acid. The purified product was obtained in a yield of 28% of the original 'NaPG'- 35% of theory on the assumption that the 'NaPG' had contained 80% of non-ketonic glucuronidate. The low yield is due to incomplete extraction of the glucuronidic acid from aqueous solution with ether.

After each separation the non-ketonic fraction was extracted from the ether with sodium hydroxide solution, and from the sodium hydroxide with butanol. Total losses of 'NaPG' were reduced to a minimum by eliminating completely the water-washing of this butanolic extract, except after the final separation. Instead, the butanol was saturated with $CO_2$ before distillation to dryness, and the fraction thus obtained, contaminated with considerable quantities of sodium bicarbonate, was submitted directly to the next fractionation.

The/

The efficiency of the whole separation process and the purity of the products obtained was investigated by determining quantitatively the pregnanediol and 20-ketosteroid contents of the toluene-soluble material obtained from the original 'NaPG' and the non-ketonic sodium glucuronidate after acid hydrolysis. The methods used for these determinations have already been described (pp. 70-71). It should be noted that these methods are not highly accurate. The toluene-soluble hydrolysis product from the non-ketonic sodium glucuronidate melted, without purification, at 233-237° (corr.) and was found to contain 98% pregnanediol and 5.2% 20-ketosteroid. The corresponding figures for the toluene-soluble hydrolysis product from the original 'NaPG' were, m.p. 211-220° (corr.), 77% pregnanediol and 23% 20-ketosteroid.

The pregnanediol and 20-ketosteroid contents of the unpurified hydrolysis product obtained from the non-ketonic sodium glucuronidate indicate that the material is nearly pure pregnane-3$\alpha$:20$\alpha$-diol, a conclusion borne out by the melting point which was raised after recrystallization to 236-238° (not depressed/

depressed by admixture with an authentic sample of pregnane-3$\alpha$:20$\alpha$-diol).

These results, considered together with the results of the elementary analyses referred to previously, clearly show that the non-ketonic sodium glucuronidate is nearly pure sodium pregnane-3$\alpha$:20$\alpha$-diol glucuronidate.

In confirmation of the results obtained by Marrian and Gough (1946) with 'NaPG', it has been found that sodium pregnanediol glucuronidate takes up three molecules of water of crystallization when exposed to moist air, and that this trihydrate is converted to the anhydrous substance by drying at 137° in vacuo over $P_2O_5$, whilst in vacuo over $CaCl_2$ at room temperature it loses 1.5 $H_2O$.

Pregnane-3$\alpha$:20$\alpha$-diol glucuronidic acid was obtained from the purified sodium salt and, after purification, melted at 178-180° (corr. with decomposition and evolution of gas), and had $[\alpha]_D^{20.5°}$ -5.0 $\pm$ 0.5°. The acid which had been stored in a moist atmosphere lost 1.5 $H_2O$ in vacuo over $CaCl_2$ at room temperature, and analytical figures then obtained were in good agreement with those required for/

for the anhydrous glucuronidic acid, $C_{27}H_{44}O_8$.

Experimental

(a) Separation and purification of the non-ketonic sodium glucuronidate from 'NaPG'.

'NaPG' (1.050 g.)(m.p. 280°, corr., with decomposition and evolution of gas) was dissolved in 50 ml. of 10% (w/w) ethanolic acetic acid and heated under reflux on a boiling water bath for 1 hr. with 3 g. trimethylammoniumacetohydrazide chloride. After cooling under a tap and then in a freezing mixture, the solution was transferred to a chilled separating funnel with 500 ml. of chilled water, and 250 ml. of chilled ether was added. After shaking, the aqueous layer was run off and re-extracted seven times as quickly as possible with 250 ml. portions of chilled ether. The ethereal extracts were combined and extracted once with 100 ml. of 0.5 N NaOH and three times with 300 ml. portions of 0.1 N NaOH. The combined NaOH extracts were then extracted six times with a total volume of 2 l. butanol, and the combined butanolic extracts were allowed to stand in a separating funnel until completely clear. After running/

running off the small aqueous layer that separated, the butanolic extract was saturated with $CO_2$ and evaporated to dryness under reduced pressure.

The material so obtained was subjected to two further 'Girard' separations in succession in a similar manner. In these separations, however, the amount of reagent T used was reduced to 2.5 g. and the volume of ethanolic acetic acid to 30 ml. The volumes of water, ether and NaOH solution, but not of butanol, were correspondingly reduced. The final butanolic extract obtained after the third separation was washed twice with one twelfth its volume of water before evaporating to dryness. The yield was 0.436 g.

Two batches of approximately 1 g. of 'NaPG' were worked up in the manner described. The non-ketonic fractions obtained after three successive separations were separately purified by dissolving in the minimum amount of 50% (v/v) aqueous acetone, filtering, and precipitating with ten volumes of dry acetone. After standing overnight in the refrigerator, the supernatant solution was carefully decanted through the filter, the precipitate filtered/

filtered with suction, and washed with cold dry acetone. This process was repeated, and the fractions so obtained were combined and subjected to one further acetone precipitation. The yields obtained during this purification procedure are shown in Table 10.

Table 10.

Yields at various stages in the purification of sodium pregnanediol glucuronidate.

| | Batch 1 g. | Batch 2 g. |
|---|---|---|
| Starting material | 0.986 | 1.050 |
| After 3 successive Girard separations | 0.427 | 0.436 |
| After first acetone precipitation | 0.373 | 0.370 |
| After second acetone precipitation | 0.344 | 0.305 |

The combined purified non-ketonic material weighed 0.649 g. It was dissolved in 34 ml. 50% aqueous acetone, filtered, and 340 ml. of dry acetone were added. After standing 12 hr. in the refrigerator, the precipitate was filtered off, washed with cold/

cold dry acetone and dried at room temperature, in vacuo, over $CaCl_2$. A total yield of 0.571 g. from 2.036 g. 'NaPG' was obtained. The purified non-ketonic sodium glucuronidate gave a virtually negative Zimmermann reaction on 5 mg. and a strongly positive Tollens reaction for glucuronic acid. It melted at 283.5-284.5° (corr., with decomposition and evolution of gas) after preliminary shrinkage at 282.5° and darkening at 283°. The 'NaPG' used as starting material melted at 279.5-280.5° (corr., with decomposition and evolution of gas).

Water of crystallization

After exposure to a moisture saturated atmosphere for 15 hr., the non-ketonic sodium glucuronidate lost 9.6% of its weight on drying at 137° over $P_2O_5$ in vacuo for 10 hr. On re-exposure to the moisture saturated atmosphere, uptake of water represented 9.9% of its final weight. The calculated water content for $C_{27}H_{43}O_8Na,3H_2O$ is 9.4%.

On drying the fully hydrated compound in vacuo over $CaCl_2$ at room temperature, it lost one half of the water of crystallization originally present. The water content of the $CaCl_2$ dried compound was found/

found to be 4.9% and 4.4%; the calculated figure for $C_{27}H_{43}O_8Na,1.5H_2O$ is 4.9%.

| | |
|---|---|
| (a) Wt. after exposure to moisture-saturated atmosphere for 15 hr. | 0.1735 g. |
| (b) Wt. after drying to constant wt. in vacuo over $CaCl_2$ at room temp. | 0.1649 g. |
| (c) Wt. after drying 10 hr. in vacuo over $P_2O_5$ at 137° | 0.1568 g. |
| (d) Wt. after re-exposure to moist atmosphere for 24 hr. | 0.1741 g. |
| (e) Wt. after re-drying as in (b) | 0.1641 g. |

Carbon, hydrogen and sodium analyses on the non-ketonic sodium glucuronidate.

(i) 2.389 mg. of the anhydrous compound, dried at 137° for 10 hr. in vacuo over $P_2O_5$, gave 1.853 mg. $H_2O$, 5.319 mg. $CO_2$, 0.253 mg. $Na_2CO_3$ and 0.020 mg. unburnt carbon.

The fully hydrated compound found to contain 9.5% moisture was used in these analyses:

(ii) 3.840 mg. ( ≡ 3.475 mg. anhydrous compound) gave 2.865 mg. $H_2O$, 7.844 mg. $CO_2$ and 0.375 mg. $Na_2CO_3$.

(iii) 4.089 mg. ( ≡ 3.701 mg. anhydrous compound) gave 3.131 mg. $H_2O$, 8.346 mg. $CO_2$ and 0.404 mg. $Na_2CO_3$.

Found/

Found: (calculated for anhydrous compound)
C, 62.7, 62.8, 62.7%; H, 8.4, 8.1, 8.3%;
Na, 4.4, 4.7, 4.7%.

Calculated: C, 62.5%; H, 8.4%; Na, 4.4%.

(b) Hydrolysis of 'NaPG' and of the non-ketonic sodium glucuronidate.

Samples of approximately 0.1 g. of 'NaPG' and of the purified non-ketonic sodium glucuronidate were each treated as follows:- The sample was suspended in 250 ml. of water and 65 ml. of toluene was added. The mixture was brought to the boil under reflux, when 25 ml. conc. HCl was added, and the boiling continued for 10 min. The solution was cooled, transferred to a separating funnel, and the aqueous layer run off, and extracted four times more with 65 ml. toluene. (The large number of extractions was necessitated by the unexpected precipitation of material during hydrolysis in the flask containing the purified glucuronide. Since three extractions failed to dissolve the precipitate, the solution was transferred back to the original flask and heated to boiling point with the fourth lot of toluene. This procedure was repeated exactly/

exactly with the 'NaPG' to maintain uniformity of treatment).

The combined toluene extracts, 325 ml., were washed once with 50 ml. of 5% $NaHCO_3$ and twice with water, and evaporated to dryness. The 'NaPG' (99.8 mg.) yielded 54.2 mg. of toluene-soluble hydrolysis product and the non-ketonic sodium glucuronidate (103.2 mg.) yielded 62.0 mg. of toluene-soluble hydrolysis product. Approximately 5 mg. of each hydrolysis product was weighed out accurately into a 10 ml. volumetric flask, dissolved in ethanol, and made up to the mark. Suitable aliquots of these solutions were taken for estimation of pregnanediol and 20-ketosteroids. The results obtained are summarized in Table 11.

Table 11 /

Table 11.

Pregnanediol and pregnanolone contents of the toluene-soluble hydrolysis products of 'NaPG' and of non-ketonic sodium glucuronidate.

| | 'NaPG' | Non-ketonic sodium glucuronidate |
|---|---|---|
| Wt. of toluene-soluble hydrolysis product in 10 ml. ethanol | 5.032 mg. | 4.858 mg. |
| Aliquots taken for 20-ketosteroid determinations | 2 ml. | 4 ml. |
| Toluene-soluble hydrol. product/aliquot | 1.016 mg. | 1.943 mg. |
| Pregnanolone found/aliquot | 0.238 mg. | 0.101 mg. |
| Pregnanolone content | 23.4% | 5.2% |
| Aliquots taken for pregnanediol determination | 0.5 ml. | 0.5 ml. |
| Toluene-soluble hydrol. product/aliquot | 0.254 mg. | 0.243 mg. |
| Pregnanediol found/aliquot | 0.197 mg. | 0.238 mg. |
| Pregnanediol content | 77.6% | 97.9% |

After drying for 22 hr. at 80° over $P_2O_5$, the toluene-soluble fraction from the hydrolysis of the non-ketonic sodium glucuronidate melted as follows:- softening starts at 222°, melting point 226-230° (corr., 233-237°). For the hydrolysis product of 'NaPG', similarly dried, the figures were: shrinkage at 197°, melting point 205-215° (corr., 211-220°).

After one recrystallization from absolute ethanol, the material obtained from the hydrolysis of the non-ketonic sodium glucuronidate melted at 236-238°(corr.); mixed with authentic pregnane-3α, 20α-diol (m.p. 235-237°, corr.), the melting point was 235-238°.

(c) Preparation of pregnanediol glucuronidic acid from its sodium salt.

0.174 g. of the purified non-ketonic sodium glucuronidate was dissolved in 60 ml. of water-saturated butanol with warming. The solution was acidified with 2 ml. of glacial acetic acid, transferred to a separating funnel, and washed three times with 5 ml. portions of water, before evaporating to dryness under reduced pressure.

The residue (0.166 g.) was heated on a boiling water/

water bath with 5 ml. of water, and the mixture filtered hot. The solid on the filter was washed back into the flask with hot ethanol, and taken to dryness at reduced pressure; the insoluble material was then leached twice more, in the same way, with 5 ml. portions of water. The final water-leached residue dissolved easily in 20 ml. of hot 25%(v/v) aqueous ethanol; the hot solution was filtered and evaporated under reduced pressure to the point of incipient crystallization. It was allowed to cool slowly to room temperature and then set aside in the refrigerator overnight.

The well-formed but slightly buff-coloured crystals that separated were collected by centrifugation, washed once in the centrifuge tube with 10 ml. of cold water and dried at room temperature over $P_2O_5$, in vacuo. A yield of 0.129 g. of material melting at 178-180° (corr., with decomposition and evolution of gas) was obtained.

Material weighed after exposure to a moist atmosphere (2.160 mg.) was dried to constant weight in vacuo over $CaCl_2$ at room temperature. The weight/

weight loss observed was 0.122 mg. or 5.6%. Similarly treated 10.497 mg. lost 0.581 mg. or 5.5%. Calculated for $C_{27}H_{44}O_8$, 1.5 $H_2O$: 5.2% $H_2O$. The melting point was unchanged after drying.

A solution of the anhydrous substance (9.916 mg.) in 0.5 ml. ethanol gave a rotation of -0.049 (standard error 0.0054°) in a 0.5 dm. tube. Hence

$$[\alpha]_D^{20.5^\circ} = -5.0 \pm 0.5^\circ.$$

2.038 mg. dried at room temperature over $CaCl_2$ in vacuo for 70 hr. gave 4.866 mg. $CO_2$ and 1.655 mg. $H_2O$.

2.378 mg., similarly dried, gave 5.688 mg. $CO_2$ and 1.923 mg. $H_2O$.

Found: C, 65.1 and 65.2%; H, 9.1 and 9.1%.
Calculated for $C_{27}H_{44}O_8$: C, 65.3%; H, 8.9%.

Summary /

Summary.

1. A study has been made of various possible methods of separating the non-ketonic sodium glucuronidate from the 'sodium pregnanediol glucuronidate' isolated from human pregnancy urine.

2. The separation of almost pure sodium pregnane-3α:20α-diol glucuronidate by means of trimethylammoniumacetohydrazide chloride (the reagent T of Girard and Sandulesco) is described.

3. Pregnane-3α:20α-diol glucuronidic acid has been prepared from its purified sodium salt.

## Section IV.

## SEPARATION OF THE KETONIC SODIUM GLUCURONIDATE FROM THE 'SODIUM PREGNANEDIOL GLUCURONIDATE' ISOLATED FROM HUMAN PREGNANCY URINE BY THE VENNING METHOD.

A. Introduction.

B. Results.

C. Experimental.

D. Discussion.

E. Summary.

A. Introduction.

When the problem of separating pure sodium pregnanediol glucuronidate was first undertaken, it was hoped that the method used to accomplish this separation would also make possible the isolation of the sodium pregnanolone glucuronidate which was presumed to be the other principal constituent of 'NaPG'.

The method which finally proved successful in separating the almost pure non-ketonic glucuronidate should obviously be applicable to the isolation of the ketonic complex. Unfortunately, the same two reasons which made the procedure inefficient in the isolation of the non-ketonic fraction also operate in this case: the ketonic fraction will be contaminated with pregnanediol glucuronide, due to the low solubility of the glucuronidic acid in ether; and the necessity of working in acid solution will cause hydrolysis of the Girard derivative and accompanying loss of ketonic material. In fact, the whole process is one of concentration rather than of separation. Nevertheless, provided sufficient starting material is available, by subjecting/

subjecting the original ketonic fraction to several further fractionations, it should be possible to obtain a ketonic sodium glucuronidate fraction almost free from non-ketonic material.

B. Results.

The isolation of a relatively pure ketonic sodium glucuronidate from 'NaPG' has proved to be considerably more difficult than was the isolation of the corresponding non-ketonic fraction. This was partly due, no doubt, to the fact that the ketonic fraction forms only 20% of the starting material; but the difficulties encountered have also been caused by the fact that, whereas the non-ketonic fraction, obtained by ether extraction, consists of relatively pure non-ketonic sodium glucuronidate, the ketonic fraction, obtained by extraction of the reaction mixture with butanol, contains a very much larger proportion of impurities. It has not been determined whether the bulk of these impurities arise from the 'NaPG', or from the reagents used; but the repeated purifications necessary to eliminate them caused considerable losses of the/

the final ketonic fraction.

The first attempts to isolate the ketonic sodium glucuronidate made use of the ketonic fractions discarded during the isolation of the non-ketonic glucuronide. The combined 'ketonic' fractions from the three successive Girard separations were used as starting material. After two further separations, the non-ketonic fraction being rejected in each case, the purified ketonic sodium glucuronidate melted at 257-260° (corr., decomposition and evolution of gas), gave a strongly positive Tollens' reaction for glucuronic acid, and an intense brick-red colour in the Zimmermann test. The yield was 6% of the original 'NaPG' but analysis suggested that the product was contaminated with inorganic material. The toluene-soluble material obtained on acid hydrolysis was estimated to contain 23% of pregnanediol and 81% of 20-ketosteroids. It melted at 127-149° (corr.), but after purification the melting point was raised to 145-148.5° (corr.)(not depressed by admixture with authentic pregnan-3α-ol-20-one).

In a second attempt to isolate the ketonic sodium glucuronidate, approximately 7.8 g. of crude 'NaPG' /

'NaPG' was worked up in two batches; it is impossible to assess the recovery as residues from previous separations were added at various stages. The final ketonic fractions, all of which had undergone at least four successive fractionations, weighed 0.509 g. After purification by repeated precipitation from aqueous acetone with dry acetone, and recrystallization from 90% ethanol, 0.083 g. of first grade ketonic sodium glucuronidate was obtained. It melted at 266-267.5° (corr., decomposition and evolution of gas) and gave a strongly positive Tollens' test. Analyses were in good agreement with the requirements for $C_{27}H_{41}O_8Na$.

The interpretation of the moisture content figures is not obvious; exposed to moist air, the ketonic sodium glucuronidate was found to contain 8.9% of water. The theoretical figures for $C_{27}H_{41}O_8Na$, 2.5 $H_2O$ and $C_{27}H_{41}O_8Na$, 3 $H_2O$ are 8.0 and 9.5% respectively. The moisture content after drying in vacuo at room temperature over $CaCl_2$ suggests that under these conditions the ketonic sodium glucuronidate exists as the monohydrate.

The compound was subjected to acid hydrolysis, and the neutral toluene-soluble hydrolysis product estimated/

estimated for pregnanediol and 20-ketosteroids by the methods described in the previous section (p.70); the apparent pregnanediol content was found to be approximately 7%, and the 20-ketosteroid content 92%.

The ketonic glucuronidic acid was prepared from the purified sodium salt. Crystallized from aqueous ethanol, it melted at 191-193° (corr., decomposition and evolution of gas), and had $[\alpha]_D^{20°}$ + 50.4° (mean value).

C. Experimental.

The preparation of the starting material, the conditions under which the Girard derivative was formed and the non-ketonic fraction extracted, and the estimation procedures employed have already been described fully in the previous section.

Following the extraction of the non-ketonic fraction from the reaction mixture, the ketonic fraction was obtained in the following manner: The aqueous phase was acidified with concentrated HCl/

HCl (2.0 ml./100 ml. solution). After standing at room temperature for 1 hr., the mixture was made just neutral to litmus by the addition of 10 N NaOH, and then an excess of 1.0 ml. of 10 N NaOH/100 ml. solution was added. This alkaline solution was then extracted six times with a total of twice its volume of butanol, and the extract allowed to stand in a separating funnel until clear. The small aqueous layer that separated was run off; and the butanol was saturated with $CO_2$, or, after the final separation, washed with water, and evaporated to dryness under reduced pressure.

Ketonic sodium glucuronidate isolated from the fractions rejected during the purification of sodium pregnanediol glucuronidate.

In the first attempt to isolate the ketonic glucuronide, the ketonic fractions obtained from the three successive Girard separations directed to isolating the non-ketonic glucuronide were combined, and subjected to two further separations. The material was worked up in two batches, corresponding to the two batches of non-ketonic glucuronide from which the starting material was derived. The butanolic extracts containing the ketonic fractions after the final separations were washed twice with/

with 1/12 volume of water before evaporating to dryness. The yields obtained in this experiment are shown in Table 12.

Table 12.

Yields obtained at various stages in the purification of the ketonic sodium glucuronidate.

| | Batch 1 g. | Batch 2 g. |
|---|---|---|
| Wt. of original 'NaPG' | 0.986 | 1.050 |
| Approx. wt. of combined ketonic fractions | 0.6 | 0.6 |
| Wt. of ketonic fraction after two further separations | 0.235 | 0.360 |
| Wt. after precipitation from 15 ml. of 50% acetone | 0.090 | 0.137 |
| Wt. after reprecipitation from 10 ml. of 50% acetone | 0.069 | 0.114 |

The two purified ketonic fractions were combined (0.178 g.), dissolved in 15 ml. 50% aqueous acetone, filtered, and precipitated with 150 ml. dry acetone; the product (0.131 g.) melted at 259/

259-262° (corr., decomposition and evolution of gas).

1.994 mg., dried at 137° over $P_2O_5$ in vacuo for 10 hr., gave 1.449 mg. $H_2O$, 4.193 mg. $CO_2$ and 0.271 mg. $Na_2CO_3$; 2.470 mg., similarly dried, gave 1.782 mg. $H_2O$, 5.201 mg. $CO_2$ and 0.333 mg. $Na_2CO_3$.

Found: C, 58.8, 58.9%; H, 8.1, 8.1%; Na, 5.9,5.8%.
Calculated for $C_{27}H_{41}O_8Na$: C, 62.8%; H, 8.0; Na, 4.5%.

101.4 mg. were suspended in 250 ml. of water and 65 ml. of toluene were added. The mixture was brought to the boil under reflux, 25 ml. of conc. HCl added, and boiling continued for 10 min. The flask was cooled under the tap, and the mixture transferred to a separating funnel. The aqueous phase was run off, and extracted four times more with 65 ml. portions of toluene. The combined toluene extracts were washed once with 50 ml. of 5%(w/v) $NaHCO_3$, twice with 50 ml. of water, and evaporated to dryness. The toluene-soluble hydrolysis product weighed 62.0 mg. and melted at 127-147° (corr.). Attempts to crystallize it from hexane yielded an amorphous product; moreover a/

a considerable amount of material insoluble in boiling hexane was present. 38.6 mg. were purified by chromatography on an alumina column (6 x 1.3 cm.). The ketonic aglucone was put on to the column dissolved in benzene; elution with ether gave 26.5 mg. of material, which, after two crystallizations from hexane, melted at 145-148.5° (corr.) and did not depress the melting point of authentic pregnan-3α-ol-20-one, m.p. 147-149.5°.

Ketonic sodium glucuronidate after four successive Girard separations.

The separations were carried out in the manner already described. The butanol extracts containing the final ketonic fractions were washed four times with 1/10 volume of water.

From approximately 5.5 g. of crude 'NaPG', after four successive separations, 0.420 g. of ketonic material was obtained. This would not dissolve completely on refluxing with 30 ml. of 50% aqueous acetone. The hot solution was filtered, and, on addition of 300 ml. of dry acetone to the filtrate, a precipitate weighing 0.207 g. was obtained. A further 0.038 g. was obtained by taking/

taking the mother liquor to dryness and repeating the acetone precipitation, using 10 ml. of 50% acetone.

The once acetone-precipitated product(245 mg.) was refluxed with 10 ml. of 50% acetone; the hot solution was decanted through a Hirsch filter funnel, and the insoluble material was refluxed with a further 5 ml. of aqueous acetone, which was then used to wash the filter. Approximately 40 mg. were still undissolved by the 50% acetone. On addition of ten volumes of dry acetone to the combined filtrate and washings, the precipitate weighed 153 mg., and melted at 195-220° (uncorr.). On recrystallization from 40 ml. of 90% ethanol, 56 mg. of crystals, melting at 253-256° (uncorr.) were obtained (Fraction P1). From the mother liquor a second crop of 24 mg. of crystals melting sharply at 259-260° (uncorr.) were obtained (P2). Fraction P1, combined with the mother liquor of P2, after recrystallization from 90% ethanol, gave 27 mg. of crystals melting at 257-257.5° (uncorr.)(P3). The remainder of the twice acetone-precipitated material was added to the second batch of ketonic sodium glucuronidate.

Crude 'NaPG'(2.30 g.) was subjected to three successive Girard separations. To the ketonic fraction (0.20 g.) thus obtained, 0.24 g. of ketonic material from previous experiments, which had already undergone three successive separations, was added, and the whole subjected to a final fourth separation. The ketonic fraction weighed 0.302 g. One acetone precipitation, from 12 ml. of 50% acetone, yielded 196 mg., melting at 251-253° (uncorr.). Recrystallization from 25 ml. of 90% ethanol raised the melting point to 253-254° (uncorr.). This fraction (93 mg.) was combined with fraction P3 (27 mg.); after recrystallization from 90% ethanol, 73 mg. of crystals, melting at 257-258° (uncorr.) were obtained (Fraction Q1. ). From the mother liquors, after repeated acetone precipitation, and recrystallization from aqueous ethanol, two further crystalline fractions were obtained: Q2, m.p. 256-257° (uncorr.), 16 mg.; and Q3, m.p. 258-260° (uncorr.), 19 mg.

Fractions P2, Q1, Q2 and Q3 were combined (135 mg.), refluxed with 15 ml. 50% acetone, and the solution filtered with suction. The filter was not washed since the insoluble material was gummy/

gummy and slightly yellow in appearance. Dry acetone (150 ml.) was added to the filtrate; a very fine 'silky' precipitate formed. After standing for several hours in the refrigerator, the precipitate was filtered off and washed with ice-cold, dry acetone. The product weighed 83 mg.; it melted at 266-267.5° (corr., decomposition and evolution of gas). From the mother liquors a further 34 mg. of material melting at 264-266°(corr.) were recovered.

Melting points of the various glucuronidate fractions.

These figures are given to show how small an effect on the melting point is produced by gross contamination with a closely related glucuronidate. Since accurate corrections are not available for all the temperatures, the melting points are given uncorrected.

| | |
|---|---|
| Original 'NaPG' ..... | 269-270° |
| Non-ketonic sodium glucuronidate | 273-274° |
| Ketonic sodium glucuronidate.... | 256-257.5° |
| Non-ketonic and ketonic sodium glucuronidates, mixed approx. 1:1 ..... | 265-267° |

Carbon/

Carbon, hydrogen and sodium analyses.

2.858 mg. of the first crop crystals, dried at 80° over $P_2O_5$, in vacuo, for 10 hr., gave 2.050 mg. $H_2O$, 6.564 mg. $CO_2$ and 0.270 mg. $Na_2CO_3$.

2.531 mg., similarly dried, gave 1.817 mg. $H_2O$, 5.817 mg. $CO_2$ and 0.235 mg. $Na_2CO_3$.

Found: C, 62.6, 62.7; H, 8.0, 8.0; Na, 4.1, 4.3%.
Calculated for $C_{27}H_{41}O_8Na$: C, 62.8; H, 8.0; Na, 4.5%.

When the sodium was determined separately as sodium sulphate, the following results were obtained:

2.335 mg. dry compound gave 0.330 mg. $Na_2SO_4$; 1.804 mg. dry compound gave 0.260 mg. $Na_2SO_4$.

Found: Na, 4.6, 4.7%.

Moisture content of ketonic sodium glucuronidate.

| | |
|---|---|
| (a) Wt. of sample, fully hydrated | 11.196 mg. |
| (b) Wt. after drying to constant wt. over $CaCl_2$ at room temperature, in vacuo | 10.600 mg. |
| (c) Wt. after drying over $P_2O_5$ at 80°, in vacuo, for 10 hr. | 10.215 mg. |
| (d) Wt. after re-equilibration with water-saturated atmosphere | 11.211 mg. |
| (e) Wt. after redrying, as in (b) | 10.630 mg. |
| (f) Wt. after re-exposure to moist atmosphere | 11.230 mg. |

Found: For fully hydrated compound, $H_2O$, 8.8,8.9,9.0%.

For $CaCl_2$ dried compound, $H_2O$, 3.9, 3.6%.

Calculated for $C_{27}H_{41}O_8Na,H_2O$: 3.4% $H_2O$.

$C_{27}H_{41}O_8Na,1.5\ H_2O$: 5.0% $H_2O$.

$C_{27}H_{41}O_8Na,2.5\ H_2O$: 8.0% $H_2O$.

$C_{27}H_{41}O_8Na,\ 3\ H_2O$ : 9.5% $H_2O$.

Hydrolysis of ketonic sodium glucuronidate.

The fully hydrated compound (37.2 mg. ) was hydrolysed with hydrochloric acid, and the toluene-soluble hydrolysis product (19.2 mg.) extracted, in the manner already described. A known amount of the hydrolysis product, weighed after drying 10 hr. at 110°, was dissolved in ethanol and aliquots were taken for determination of pregnanediol and of 20-ketosteroids.

4.975 mg. of the toluene-soluble hydrolysis product gave a reading in the sulphuric acid colour reaction equivalent to 0.550 mg. of pregnane-3α;20α-diol. Since 4.983 mg. of authentic pregnane-3α-ol-20-one gave a colour equivalent to that produced by 0.180 mg. pregnane-3α,20α-diol, the apparent pregnanediol content is approximately 7.4%.

In/

In the Zimmermann reaction, 0.298 mg. of the hydrolysis product gave a colour equivalent to that produced by 0.275 mg. of authentic pregnan-3α-ol-20-one, indicating a pregnanolone content of 92%.

Preparation of the ketonic glucuronidic acid.

The ketonic sodium glucuronidate (29.7 mg.) was dissolved with slight warming in 40 ml. of water-saturated butanol. Glacial acetic acid (1 ml.) was added and the solution was transferred to a separating funnel. The butanol was washed four times with 5 ml. portions of water, both layers being allowed to clear completely at each washing, and evaporated to dryness under reduced pressure. The residue, dried over $CaCl_2$ and NaOH at room temperature, in vacuo, weighed 23.5 mg.

The product was leached with 1 ml. of cold water, the water filtered off through a sintered-glass filter, and the insoluble material washed back into the flask with hot ethanol and taken to dryness. The process was repeated once with 1 ml. of boiling water. The water-washed residue was dissolved/

dissolved in 3 ml. of 25% (v/v) ethanol and filtered. The volume of the filtrate was gradually reduced until the point of incipient crystallization was reached, when the solution was allowed to cool slowly, first to room temperature, and then in the refrigerator. The precipitate was almost pure white and consisted of minute, star-shaped clusters of needle-like crystals; it was collected by centrifugation, washed once with 1 ml. of water, in the centrifuge tube, and dried in vacuo over $CaCl_2$. The product weighed 17.2 mg. and melted at 191-193° (corr., decomposition and evolution of gas).

2.506 mg. (dried over $CaCl_2$ at room temperature, in vacuo, to constant weight) gave 5.802 mg. $CO_2$ and 1.942 mg. $H_2O$.

Found: C, 63.1; H, 8.7%.

Calculated for $C_{27}H_{42}O_8,H_2O$: C, 63.0; H, 8.6%.

Specific rotation of ketonic glucuronidic acid.

7.010 mg., dried over $P_2O_5$ at 80° in vacuo for 10 hr., dissolved in 0.4886 ml. ethanol gave a rotation of + 0.353° (standard error 0.0033 ) in a 0.5 dm. tube. Hence $[\alpha]_D^{20.5} = + 49.2° \pm 0.4°$.

6.057 mg., similarly dried, dissolved in 0.4856 ml. of ethanol gave a rotation of + 0.323° (standard error 0.0051) in a 0.5 dm. tube. Hence

$$[\alpha]_D^{20.5} = + 51.8° \pm 0.8°.$$

D./

D. Discussion.

Although the ketonic sodium glucuronidate fraction obtained is obviously not pure sodium pregnanolone glucuronidate, this compound probably represents about 90% of the final product. In spite of the rather close agreement which exists between the apparent pregnanediol content (7%) and the apparent non-20-ketosteroid content (8%) of the hydrolysis products, it is doubtful whether sodium pregnanediol glucuronidate is still present to this extent. The possible presence of the glucuronide of another ketone, less chromogenic than pregnanolone in the Zimmermann test, but giving a greater colour with concentrated sulphuric acid, must be borne in mind. The behaviour of the sodium pregnanolone glucuronidate fraction during purification suggests that other very similar compounds are present in the final ketonic fraction; at least one of these contaminants is relatively insoluble in 50% acetone, and thus does not resemble sodium pregnanediol glucuronidate.

It/

It is interesting to note that although a mixture of the two glucuronidates cannot be separated by repeated 'acetone precipitation', the ketonic sodium glucuronidate, when relatively free from non-ketonic material, appears to be considerably less soluble in 50% acetone and more soluble in 95% acetone than the pure sodium pregnanediol glucuronidate.

The contention that the ketonic sodium glucuronidate isolated is relatively free from the pregnanediol complex is supported by the rotation of the free glucuronidic acid. The observed $[\alpha]_D$ of 50.5° (average) gives a molecular rotation, $[M]_D$ of + 249°. Since the $[M]_D$ of pregnane-3α:20α-diol is + 83° and the $[M]_D$ of pregnan-3α-ol-20-one + 350°, the molecular rotation contribution of replacing the C 20 hydroxyl group by a carbonyl group is + 267°. The $[M]_D$ of pregnane-3α:20α-diol glucuronidic acid, calculated from the observed value for $[\alpha]_D$, is - 25°, so the pregnanolone glucuronidic acid should have an $[M]_D$ of about + 240°. That the $[M]_D$ value found, though/

though in reasonably good agreement with the calculated value, is somewhat higher than might be expected, confirms the belief that the ketonic sodium glucuronidate contains little sodium pregnanediol glucuronidate, since this contaminant would lower the rotation.

E. Summary.

1. The separation of a ketonic sodium glucuronidate from the 'sodium pregnanediol glucuronidate' isolated from human pregnancy urine by the method of Venning has been described. Analytical data indicate that sodium pregnan-3α-ol-20-one glucuronidate forms approximately ninety per cent. of this fraction.

2. The possibility that the remaining material consists of glucuronidates other than that of pregnane-3α:20α-diol is discussed.

## SECTION V.

## THE ESTIMATION OF PREGNAN-3α-OL-20-ONE

A. Introduction

B. The estimation of pregnanolone by means of the Zimmermann reaction.

Results
Experimental
Discussion

C. The estimation of pregnanolone by reduction with lithium aluminium hydride.

Results
Experimental
Discussion

D. Summary

A. Introduction

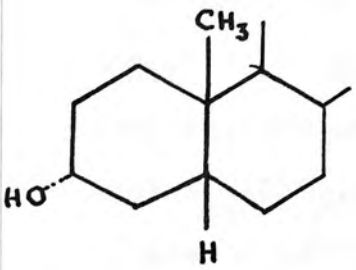
During the course of the separation of the non-ketonic and ketonic sodium glucuronidates, a considerable number of estimations of 20-keto-steroids was carried out. It became clear that the modification of the Zimmermann reaction developed by Callow, Callow and Emmens (1938) for the estimation of 17-ketosteroids, unchanged except by the use of different filters, was not entirely suitable for determining 20-ketosteroids. While on some occasions the colours obtained with known amounts of pregnanolone were perfectly satisfactory, at other times the lack of concordancy among the results made them completely meaningless.

The reaction of pregnanolone with m-dinitro-benzene in ethanolic potassium hydroxide solution has therefore been studied under various conditions, with the object of increasing the accuracy of 20-ketosteroid assays on future purified fractions. Although the method evolved is of value for this purpose, it is not suited to measuring 20-keto-steroids in urinary extracts. While it would probably/

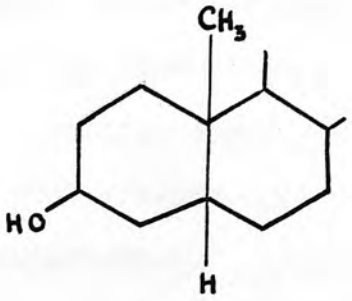
probably be possible to estimate 20-ketosteroids in the presence of 17-ketosteroids, in pure solution, making use of the slightly different absorption maxima of the colours developed, and of the different rates of colour development, this would not be possible in urinary extracts, owing to the great preponderance of the more highly chromogenic 17-ketosteroids and to interference by other chromogens.

It would, nevertheless, be of interest to be able to estimate the excretion of 20-ketosteroids. Following the administration of progesterone, the pregnanediol excreted in the urine represents, in normal subjects, less than 20% of the administered dose (Sommerville and Marrian, 1950). Estimation of the urinary 20-ketosteroids after progesterone administration would indicate whether any considerable amount of the administered progesterone is excreted in the less fully reduced state.

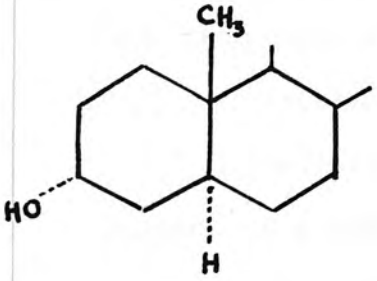
Of the four isomeric pregnan-3-ol-20-ones which might theoretically be derived from progesterone (I-IV), three have been isolated from/



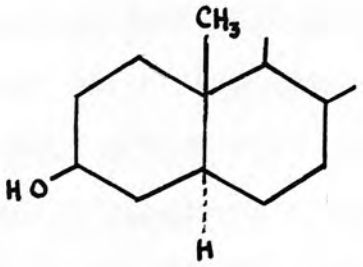

I. Pregnan-3α-ol-




II. Pregnan-3β-ol-




III. Allopregnan-3α-ol




IV. Allopregnan-3β-ol-

from human pregnancy urine: pregnan-3α-ol-20-one (I) by Marker and Kamm (1937); allopregnan-3α-ol-20-one (III) by Marker, Kamm and McGrew (1937); and allopregnan-3β-ol-20-one (IV) by Pearlman, Pincus and Werthessen (1942).

Pregnan-3α-ol-20-one glucuronide has been shown by Marrian and Gough (1946) invariably to accompany pregnanediol glucuronide in the 'sodium pregnanediol glucuronidate' isolated by the method of/

of Venning and Browne (1936); since 'sodium pregnanediol glucuronidate' has been shown to be derived from progesterone (Venning, Henry and Browne, 1937), it follows that pregnan-3α-ol-20-one is also a metabolite of progesterone. This deduction is confirmed by the work of Dorfman, Ross and Shipley (1948), who isolated pregnan-3α-ol-20-one from the urine of a male subject following, but not before, progesterone administration.

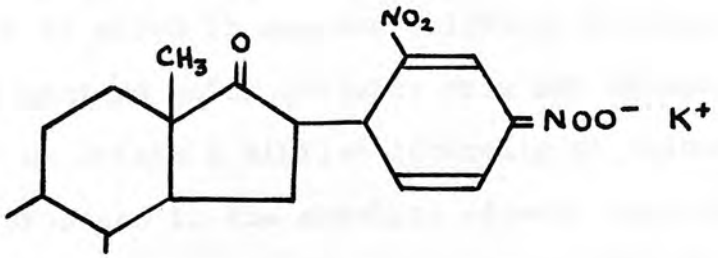
Kyle (1950) has recently proved that progesterone is a precursor of allopregnane-3α:20α-diol; this finding lends support to the belief, previously held, that the allopregnanolones of pregnancy urine are also derived from progesterone.

The method of estimating pregnanediol described by Sommerville, Gough and Marrian (1948) is one of the more accurate assays available for urinary steroids. Since this method also determines allopregnane-3α:20α-diol, it seems that it might be possible to estimate the urinary pregnanolones by reduction to the corresponding pregnanediols, the 'pre-formed' or true pregnanediol and 'total'/

'total' pregnanediol (after reduction) being estimated separately.

B. The estimation of pregnanolone by means of the Zimmermann reaction.

The reaction for 17-ketosteroids described by Zimmermann (1935, 1936) depends on the colour developed by such compounds in the presence of m-dinitrobenzene in alcoholic potassium hydroxide solution. It is rather non-specific, the chromogenic group being $-CH_2-CO-$; according to Zimmermann, the colour is due to the compound formed by the addition of dinitrobenzene to the activated methylene group:




Callow, Callow and Emmens (1938) first placed the reaction with 17-ketosteroids on a sound quantitative basis. It is not proposed to consider in detail the many other modifications of the Zimmermann reaction/

reaction which have been published. All have been designed for determining steroids with a ketonic group at C 17. The main differences lie in the proportions of ethanol and water present in the reaction mixture, but the concentration of potassium hydroxide used, and the time allowed for colour development also vary.

In the method of Callow et al. (1938) steroid, dinitrobenzene, and potassium hydroxide are all dissolved in absolute ethanol. After colour development for 60 minutes, the reaction mixture is diluted, also with absolute ethanol. Holtorff and Koch (1940) use 95% ethanol to dissolve the steroid and the dinitrobenzene, while the potassium hydroxide is added in aqueous solution, giving a reaction mixture which contains only 63% ethanol. In order to obtain a similar intensity of colour to that produced in the absolute ethanol reaction mixture, the concentration of potassium hydroxide has to be doubled. The colour is developed for 45 minutes, and dilution made with 95% ethanol.

These two modifications of the dinitrobenzene reaction have been reported to be the most accurate and/

and reliable by independent groups of workers (Engstrom and Mason, 1943; Nathanson and Wilson, 1943). Because of the great convenience of using a stable reagent, aqueous potassium hydroxide has been used in all the experiments described. The aqueous ethanolic reaction mixture has the added advantage that it dissolves glucuronidate fractions as well as free steroids. Since these experiments were conducted, a method has been described for stabilizing alcoholic potassium hydroxide (Wilson and Carter, 1947). Normally this reagent is only stable for 2-3 days, but stored under nitrogen after the addition of ascorbic acid, it may be kept for several weeks. This discovery removes one of the great disadvantages of the Callow method.

Results /

Results: Method finally adopted for estimating pregnanolone.

Each estimation is carried out in duplicate as follows: 0.2 ml. of a solution of the material to be estimated in 90% or absolute ethanol (containing not more than 0.2 mg. of pregnanolone), 0.2 ml. of 2% (w/v) m-dinotrobenzene in absolute ethanol, and 0.2 ml. of 5.0 N aqueous KOH are pipetted into a small reaction tube (10 x 1.3 cm.) and mixed well. The tube is tightly stoppered with a rubber bung wrapped in tinfoil, and placed in a water bath at 25°C. ± 0.1°. The reaction is timed from the addition of the KOH. The tube is shaken after 5, 10 and 15 minutes, to ensure that all the dinitrobenzene is redissolved. After 150 minutes, 6 ml. of 95% (v/v) ethanol are run in from a burette, the tube is restoppered, and the contents mixed by repeated inversion. The colour developed is read in the Spekker photoelectric absorptiometer using Ilford spectrum blue-green (603) filters, not less than 5 minutes and not more than 25 minutes after dilution. The reading obtained is corrected for the colour given by the reagents alone, determined/

determined at the same time. It is preferable to construct the calibration curve from results obtained with pregnanolone standards run simultaneously with the unknowns.

Experimental.

All the ethanol used in the preparation of solutions and for dilution purposes was 'aldehyde-free' prepared by allowing the ethanol to stand at least one week on m-phenylenediamine (4 g./l.) before distillation over a glass bead column.

The m-dinitrobenzene was purified according to the directions of Callow et al. (1938), followed by sublimation and a further recrystallization from absolute ethanol.

The aqueous KOH solution was prepared by dissolving water-washed KOH sticks in recently boiled water, and storing the solution with precautions to prevent $CO_2$ uptake. A sample was removed, the normality determined, and the remaining solution diluted appropriately.

To avoid needless repetition, the conditions which were common to all the experiments are described/

described here, and will not be referred to again. In every case, colour development was carried out in stoppered tubes at 25°C. ± 0.1°. Readings were made using the Spekker photoelectric absorptiometer fitted with Ilford spectrum blue-green (603) filters. Unless otherwise stated the m-dinitrobenzene solution was 2% (w/v) in absolute ethanol.

Time required for optimal colour development.

Experiment 1. The reagent blank tubes were set up, in duplicate, containing 1 ml. ethanol, 1 ml. m-dinitrobenzene solution and 1 ml. 3.96 N KOH. The pregnanolone standards, also in duplicate, were identically set up, except that they also contained 0.196, 0.981 and 1.962 mg. of pregnanolone respectively. At timed intervals during the colour development, 0.5 ml. aliquots were pipetted from the tubes into 10 ml. of 95% ethanol, and the colour developed was measured. The results are shown in Table 13.

Table 13 /

Table 13.

Time-colour relationship with 4N KOH.

| Time (minutes) ⟶ | 60 | 90 | 120 | 150 | 195 |
|---|---|---|---|---|---|
| | Spekker Readings | | | | |
| Pregnanolone added, mg. | | | | | |
| 0 | 0.003 | 0.008 | 0.010 | 0.020 | 0.040 |
| 0 | 0.003 | 0.008 | 0.010 | 0.020 | 0.039 |
| Average | 0.003 | 0.008 | 0.010 | 0.020 | 0.039 |
| 0.196 | 0.009 | 0.017 | 0.025 | 0.036 | 0.054 |
| 0.196 | 0.009 | 0.018 | 0.027 | 0.039 | 0.057 |
| Corrected average [x] | 0.06 | 0.010 | 0.016 | 0.018 | 0.017 |
| 0.981 | 0.060 | 0.097 | 0.121 | 0.148 | 0.170 |
| 0.981 | 0.060 | 0.093 | 0.116 | 0.150 | 0.171 |
| Corrected average | 0.057 | 0.087 | 0.109 | 0.129 | 0.132 |
| 1.962 | 0.152 | 0.205 | 0.240 | (0.264 | 0.272 )[xx] |
| 1.962 | 0.153 | 0.207 | 0.248 | 0.278 | 0.292 |
| Corrected average | 0.150 | 0.198 | 0.234 | 0.258 | 0.253 |

x The term corrected average indicates that the average value for the corresponding reagent blank has been deducted from the average observed value.

xx Tube left unstoppered, causing evaporation losses.

Conclusion:

The time required to develop the maximum colour under these conditions is approximately 2½ hr. After longer development, the more intense colour starts to fade, while with lesser concentrations of pregnanolone the colour may still be increasing very slightly. At the two higher levels of pregnanolone, the colours at 150 min., corrected for the reagent blank, are proportional to the amount of pregnanolone present, but the low pregnanolone level gives considerably less colour than would be expected.

Experiment 2. This experiment was exactly similar to Experiment 1, except that different quantities of pregnanolone were used, and the KOH was 4.93 N.

Table 14. /

Table 14.

Time-Colour relationship with 5 N KOH.

| Minutes ——————→ | 80 | 105 | 130 | 150 |
|---|---|---|---|---|
| | Spekker readings | | | |
| Pregnanolone added, mg. | | | | |
| 0 | 0.032 | 0.038 | 0.052 | 0.064 |
| 0 | 0.034 | 0.048 | 0.057 | 0.072 |
| Average | 0.033 | 0.043 | 0.055 | 0.068 |
| 0.202 | 0.065 | 0.082 | 0.104 | 0.120 |
| 0.202 | 0.077 | 0.094 | 0.112 | 0.128 |
| Corrected average | 0.038 | 0.045 | 0.053 | 0.056 |
| 0.505 | 0.183 | 0.209 | 0.233 | 0.252 |
| 0.505 | 0.181 | 0.207 | 0.235 | - |
| Corrected average | 0.149 | 0.165 | 0.179 | 0.184 |
| 1.010 | 0.277 | 0.330 | 0.368 | 0.389 |
| Corrected average | 0.244 | 0.287 | 0.313 | 0.321 |

Conclusion/

Conclusion: Increasing the normality of the KOH from 4 to 5 does not apparently shorten the time required for complete colour development. In this experiment no measurements were carried out after 150 minutes, but the rate of colour increase appears to be levelling off between 130 and 150 minutes.

It was noted that the duplicate results obtained during such 'time-colour' experiments appeared to be definitely less scattered than those obtained in ordinary estimations when the total volume of the reaction mixture was 0.6 ml. It is possible that this is accounted for solely by the greater accuracy obtained by pipetting 1 ml. rather than 0.2 ml. volumes. On the other hand, an additional pipetting of 0.5 ml. is required. On the whole, it seems likely that another factor is involved. One widely divergent set of readings obtained in Expt. 1 was caused by evaporation while the tube was left un-stoppered. This suggested that the more concordant results might be due to the greater ratio of volume: surface area or volume:capacity of the reaction tube. The tubes originally used were Pyrex boiling tubes, 6" x 1" with ground-glass stoppers. In some of the following experiments, narrow tubes of total capacity/

capacity ca 12 ml., tapered to a point, were used. These also were fitted with ground-glass stoppers. Later, when the volume of ethanol used for the final dilution was reduced to 6 ml., small soda-glass tubes, 10 x 1.3 cm., capacity ca 7 ml., were used with rubber bungs wrapped in tinfoil. Although it is difficult to assess accurately improvements of this nature, the impression has been gained that more concordant results have been obtained.

Experiment 3. The rate of colour development after 150 minutes was tested at one level of pregnanolone only. The reagent blank tubes contained 0.2 ml. ethanol, 0.2 ml. dinitrobenzene solution, and 0.2 ml. 5.00 N KOH. The pregnanolone standards contained 0.101 mg. pregnanolone in 0.2 ml. ethanol, dinitrobenzene and KOH solutions as in the reagent blanks. After development, 6 ml. of 95% ethanol was used for dilution.

Table 15 /

Table 15.

Time-colour relationship with 5N KOH.

| | Minutes | | |
|---|---|---|---|
| | 140 | 160 | 180 |
| Reagent blank, Spekker readings | 0.048<br>0.048 | 0.052<br>0.058 | 0.062<br>0.078 |
| Average | 0.048 | 0.055 | 0.070 |
| 0.101 mg. pregnanolone, Spekker readings | 0.204<br>0.203 | 0.217<br>0.227 | 0.232<br>0.238 |
| Average | 0.204 | 0.222 | 0.235 |
| Corrected average | 0.156 | 0.167 | 0.165 |

Conclusion: The colour developed is probably fairly steady between $2\frac{1}{2}$ and 3 hr. after mixing.

Effect /

Effect of final dilution volume; and optimum time after dilution for reading colours.

Experiment 4: Equal volumes (1 ml. of each)of pregnanolone solution (1.010 mg./ml.), dinitrobenzene solution and 3.95 N KOH were mixed and the colour allowed to develop. Three 0.5 ml. samples were taken and diluted with 6, 10 and 15 ml. of 95% ethanol respectively. Readings were taken 5 min. after dilution, and then every 10 min. until 45 min. had elapsed.

Table 16.

Effect of final dilution volume and maximum interval allowable between dilution and reading.

| Dilution | 1 in 13 | 1 in 21 | 1 in 31 |
|---|---|---|---|
| Reading at 5 min. | 0.336 | 0.209 | 0.131 |
| 15 " | 0.339 | 0.209 | 0.129 |
| 25 " | 0.339 | 0.209 | 0.129 |
| 35 " | 0.342 | 0.211 | 0.131 |
| 45 " | 0.352 | 0.221 | 0.142 |
| Reading at 5 min. x dilution factor | 4.368 | 4.389 | 4.061 |

Conclusion /

Conclusion: Readings should be made within 25 minutes of dilution. The difference between the readings obtained with the 1 in 13 and 1 in 21 dilutions is not significant, since it represents only 1 Spekker unit in the readings. The value obtained after the 1 in 31 dilution is definitely low and suggests that the errors in estimating low levels of pregnanolone may possibly arise in the dilution process rather than during the colour development. In case this is so, the volume of 95% ethanol used for diluting the final reaction mixture has been reduced to 6 ml. instead of the 10 ml. previously used.

Minimum interval allowable between dilution and reading colours.

Experiment 5: Two tubes were set up, one containing 1 ml. ethanol, 1 ml. dinitrobenzene solution and 1 ml. 4.96 N KOH; in the other tube 1 ml. of pregnanolone solution (containing 1.010 mg.) replaced the ethanol. After development for 2½ hr., 0.5 ml. aliquots were diluted with 6 ml. 95% ethanol.

Table 17. /

Table 17.

Minimum interval allowable between dilution and reading colours.

| Time after dilution, min. | Spekker Reading | | |
|---|---|---|---|
| | Blank B | Standard S | S - B |
| ½ | 0.043 | 0.347 | 0.304 |
| 1½ | 0.047 | 0.368 | 0.321 |
| 2 | 0.049 | 0.378 | 0.329 |
| 3 | 0.050 | 0.381 | 0.331 |
| 5 | 0.052 | 0.387 | 0.335 |
| 6 | 0.052 | 0.388 | 0.336 |

Conclusion: The rapid increase in colour which occurs after dilution appears to be complete within 5 minutes. Readings should not be taken until this interval has elapsed, if concordant results are to be obtained.

Effect of the concentration of ethanol used for the final dilution.

Experiment 6: Two tubes were set up as described for Expt. 5, except that 3.95 N KOH was used. After colour development 0.5 ml. aliquots were diluted with 10 ml. volumes of different concentrations of aqueous ethanol, and readings were taken.

Table 18 /

Table 18.

Effect of the concentration of ethanol used for the final dilution.

| Concentration of ethanol, %. | Spekker Reading | | |
|---|---|---|---|
| | Blank B | Standard S | S - B |
| 65 | 0.014 | 0.189 | 0.175 |
| 80 | 0.017 | 0.201 | 0.184 |
| 90 | 0.015 | 0.205 | 0.190 |
| 95 | 0.019 | 0.214 | 0.195 |

Conclusion: The colour developed is dependent on the concentration of ethanol used for diluting the reaction mixture. The concentration of 95% has been chosen for use since it gives more intense colours than lower ethanol concentrations, while still not precipitating the traces of carbonate which are usually present in the KOH reagent.

Effect of altering the concentration of dinitrobenzene.

As would be expected from the results reported by other workers on the reaction with 17-ketosteroids, concentrations of m-dinitrobenzene lower/

lower than 2% give a considerably less intense colour; but it is impossible to use higher concentrations of this reagent since it would then precipitate out of the aqueous alcoholic reaction mixture.

Experiment 7: Tubes were set up containing 0.2 ml. ethanol (reagent blanks) or 0.2 ml. pregnanolone solution (0.101 mg. pregnanolone) and 0.2 ml. of the appropriate solution of dinitrobenzene. The colour reactions were started at 8-minute intervals, by the addition of 0.2 ml. of 5.00 N KOH. After $2\frac{1}{2}$ hr. the contents of the tubes were diluted with 6 ml. of 95% ethanol and mixed well. The colours were read 8 min. after dilution. The results given in Table 19 are, with the exceptions indicated, the mean of three separate determinations.

Table 19 /

Table 19

The effect of the dinitrobenzene concentration.

| | Conc. of dinitro-benzene solution added, % | Spekker Readings |
|---|---|---|
| Reagent blank | 2.0 | 0.060[x] |
| Pregnanolone (101 µg.) | 2.0 | 0.261 |
| Pregnanolone(corrected) | 2.0 | 0.201 |
| Reagent blank | 1.5 | 0.046 |
| Pregnanolone (101 µg.) | 1.5 | 0.224 |
| Pregnanolone(corrected) | 1.5 | 0.178 |
| Reagent blank | 1.0 | 0.027[xx] |
| Pregnanolone(101 µg.) | 1.0 | 0.176[xx] |
| Pregnanolone(corrected) | 1.0 | 0.149 |

x One result only.
xx Two results only.

Conclusion: To obtain the greatest intensity of colour, 2% dinitrobenzene should be used. This is desirable in view of the relatively weak chromogenicity of pregnanolone.

Effect of small variations in KOH concentration.

Experiment 8. Tubes were set up containing 0.2 ml. pregnanolone solution (0.202 mg. pregnanolone), 0.2 ml. 2% m-dinitrobenzene solution, and 0.2 ml. of 5.00 N, 4.95 N or 4.90 N KOH. The appropriate reagent blanks were also set up. Colours were developed for 2½ hr. Dilutions were made with 6 ml. of 95% ethanol. Readings given in Table 20 are the mean values for three separate determinations, unless otherwise stated.

Table 20.

Effect of small variations in KOH concentration.

| KOH N | Spekker Readings | | |
|---|---|---|---|
| | Standard S | Blank B | S - B |
| 5.00 | 0.441 | 0.062 | 0.379 |
| 4.95 | 0.439 | 0.060 | 0.379 |
| 4.90 | 0.427[x] | 0.055[xx] | 0.372 |

x Two estimations only.
xx One estimation only.

Conclusion /

Conclusion: Variations of the normality of the KOH between 4.95 and 5.05 will probably not affect the results obtained. Greater variations should not be allowed unless pregnanalone standards are to be run simultaneously with the unknowns.

Linear relationship of colour produced to pregnanolone present under the conditions recommended.

Experiment 9: The method employed has already been described in detail (p.132). The results are given in Table 21.

Table 21.

| Pregnanolone added, mg. | Spekker reading | Average Spekker reading | Average reading corrected for reagent blank |
|---|---|---|---|
| 0<br>0<br>0 | 0.060<br>0.066<br>0.061 | 0.062 | |
| 0.051 | 0.137<br>0.142<br>0.140 | 0.140 | 0.078 |
| 0.101 | 0.218<br>0.223<br>0.220 | 0.220 | 0.158 |
| 0.203 | 0.380<br>0.374<br>0.381 | 0.378 | 0.316 |

Conclusion/

Conclusion: Over the range 0.050 to 0.200 mg. the colour is proportional to the pregnanolone present.

Experiments 10 and 11: These experiments are exact repliquas of Expt. 9, conducted in order to assess the reproducibility of results on different occasions (Tables 22 and 23).

Table 22.

| Pregnanolone added, mg. | Spekker Reading (a) | Average Spekker Reading (b) | (b) corrected for reagent blank |
|---|---|---|---|
| 0 | 0.059<br>0.066 | 0.063 | |
| 0.051 | 0.145<br>0.141<br>0.147 | 0.144 | 0.081 |
| 0.101 | 0.230<br>0.238<br>0.231 | 0.233 | 0.170 |
| 0.203 | 0.390<br>0.390<br>0.390 | 0.390 | 0.327 |

Table 23 /

Table 23.

| Pregnanolone added, mg. | Spekker readings (a) | Average Spekker reading (b) | (b) corrected for reagent blank |
|---|---|---|---|
| 0<br>0 | 0.062<br>0.052 | 0.057 | |
| 0.051<br>0.051 | 0.139<br>0.142 | 0.141 | 0.084 |
| 0.101<br>0.101 | 0.231<br>0.221 | 0.226 | 0.169 |
| 0.202<br>0.202 | 0.407<br>0.421 | 0.414 | 0.357 |
| 0.505<br>0.505 | 0.778<br>0.800 | 0.789 | 0.732 |

Conclusion: The results indicate that values obtained are usually within 4% of the correct value, although on some occasions the accuracy may be considerably greater than this.

Effect /

Effect of using higher concentrations of potassium hydroxide.

A few preliminary experiments were carried out to see whether the sensitivity of the method could be increased by using higher concentrations of KOH. The use of 10 N KOH is out of the question, due to the intense colour produced by reagents alone. With 6, 7, 7.5 and 8 N KOH the reagent blanks in aqueous alcohol are still low. It is possible that the maximum colour may be developed in a shorter time using the more concentrated solutions of alkali.

Table 24 /

Table 24

The effect of using higher concentrations of potassium hydroxide.

The colour values are those given by 0.10 mg. pregnanolone in 0.6 ml. of reaction mixture, diluted with 6 ml. 95% ethanol.

| KOH · N | Spekker Reading | | | Time of colour development, min. |
|---|---|---|---|---|
| | Blank B | Standard S | S - B | |
| 5 | 0.060 | 0.225 | 0.165 | 150 |
| 6 | 0.106 | 0.329 | 0.223 | 150 |
| 7 | 0.222 | 0.499 | 0.277 | 150 |
| 7.5 | 0.121 | 0.421 | 0.300 | 100 |
| 8 | 0.160 | 0.506 | 0.346 | 100 |

Discussion:

The method described has been found to be considerably more satisfactory for pregnanolone determinations than the unmodified Callow method. While the possible error of results is still about ± 4%, within these limits they are consistent. On some occasions the accuracy has been greater than this. The advantage of estimating pregnanolone standards at the same time as unknowns must be stressed, since not only does this permit the

Spekker/

Spekker readings to be converted into pregnanolone equivalents more accurately, but at the same time an indication of the possible accuracy of the whole set of determinations is obtained.

The results obtained with higher concentrations of potassium hydroxide suggest that it might be possible by this means to develop a more sensitive and shorter method of estimating pregnanolone.

C. The Estimation of Pregnanolone by Reduction to Pregnanediol.

Reduction of pregnan-3α-ol-20-one with sodium and alcohol, or with nickel and hydrogen, gives a mixture of both the pregnanediols stereoisomeric at C 20. Pregnane-3α:20β-diol is the chief product of hydrogenation with a platinum catalyst (Marker, Kamm, Wittle, Oakwood, Lawson and Laucius, 1937). In 1947 Nystrom and Brown described the use of lithium aluminium hydride as a reducing agent: aldehydes, ketones, esters, acid chlorides and anhydrides could be reduced almost quantitatively to the corresponding alcohols on a preparative scale. This reduction has the advantage that it is simple and rapid to carry out, and requires no special apparatus. The reduction of pregnan-3α-ol-20-one with this reagent was therefore investigated in the hope that the pregnanediol formed could be estimated by the method of Sommerville et al. (1948) and thus afford a method of estimating the pregnanolone.

This proposed method of estimating pregnanolone has the possible disadvantage that whereas the various/

various components of the neutral fraction of normal urine do not interfere in the determination of pregnanediol, the various reduced derivatives may do so. With the intention of checking this possibility, the reduction products of androsterone, epiandrosterone and dehydroepiandrosterone were also prepared.

Results

The product obtained on reduction of pregnan-3α-ol-20-one by $LiAlH_4$, on two different occasions, melted at 227-230° and 220-222° respectively. (All the melting points referred to in this sub-section are corrected). It probably consisted of a mixture of pregnane-3α:20α-diol and pregnane-3α:20β-diol. After recrystallization from ethanol, a product melting at 231-233° was obtained, which, mixed with authentic pregnane-3α:20α-diol ( m.p. 235-237°) melted at 211-213°. No authentic pregnane-3α:20β-diol was available for a mixed melting point, but its melting point is given as 231-234° in the literature.

The crude reduction product, however, gave a colour in the sulphuric acid reaction almost the same/

same as that given by pregnane-3α:20α-diol. The efficiency of the reduction procedure, and the recovery obtained during the subsequent purification processes were therefore tested. The results indicate that although the reduction leads to the formation of material chromogenic with sulphuric acid in almost theoretical yields, and that this reduction is probably unaffected by the presence of neutral urine fractions, the recovery obtained during the precipitation procedure employed by Sommerville et al. (1948) is very variable. It appears as if pregnane-3α:20β-diol does not form large crystals even on slow cooling. Further cooling to 0°C. does not improve the recoveries obtained, nor does changing the precipitation mixture from 20% aqueous ethanol to 15% ethanol or 20% acetone or methanol. Tests on the neutral fraction of male urine had previously shown that after $LiAlH_4$ reduction no apparent pregnanediol was detected by the method used, although the reduction products of androsterone, epiandrosterone and dehydroepiandrosterone were/

were all chromogenic in the sulphuric acid reaction.

Androstane-3α:17-diol was tested in the precipitation procedure and it was found that at levels corresponding to 2 mg. of androsterone per 24 hr. no androstanediol was recovered. At levels corresponding to 4 mg. per 24 hr. the recovery ranged from 7.6 to 26.5% and was equivalent to a daily excretion of 0.1 to 0.3 mg. of pregnanediol.

Experimental.

Preparation of an ethereal solution of lithium aluminium hydride.

Dry ether (100 ml.)(distilled once from sodium and twice from $P_2O_5$)was placed in a dry stoppered measuring cylinder. Roughly 600 mg. of $LiAlH_4$ was weighed out rapidly and added to the ether. A brisk effervescence ensued. The cylinder was stoppered, shaken well, and allowed to stand about four hours, when the hydroxides and excess $LiAlH_4$ had settled out, leaving the supernatant solution almost clear.

The/

The molarity of the solution was determined as follows: 5 ml. were pipetted into a dry flask, 2 ml. of ether were added, followed by 2 ml. of acetone mixed with 10 ml. of ether, dropwise. Water (10 ml.) was then added and the solution titrated against 1.225 N HCl using phenolphthalein as indicator; the volume used was 0.52 ml.

$$4\ R_2CO + LiAlH_4 \longrightarrow LiAl(OCHR_2)_4$$

$$LiAl(OCHR_2)_4 + 4H_2O \longrightarrow LiOH + 4R_2CHOH + Al(OH)_3$$

One molecule of lithium aluminium hydride gives one molecule of lithium hydroxide, so the solution was 0.13 M.

Reduction of pregnan-3α-ol-20-one with lithium aluminium hydride.

Pregnanolone (100 mg.) was dissolved in 15 ml. of dry ether and transferred to a dropping funnel with a further 10 ml. of dry ether. The solution was dropped slowly into 20 ml. of 0.1 M $LiAlH_4$ solution, contained in a flask fitted with a reflux condenser. Both the condenser and the dropping funnel were protected with $CaCl_2$ tubes. After the addition of the pregnanolone solution was completed, the/

the flask was heated for 15 min. on a boiling water bath. At the end of this period, 2 ml. of acetone diluted to a volume of 10 ml. with ether were added, followed by 10 ml. of water and 20 ml. of 2N $H_2SO_4$. The contents of the flask were transferred to a separating funnel and extracted three times with ether. The combined ethereal extracts were washed once with 2N $H_2SO_4$, twice with $NaHCO_3$ and three times with water and taken to dryness.

The product weighed 95 mg. and melted at 220-222°. Part of this crude reduction product was set aside for recovery experiments. Recrystallization of 40 mg. from 2.5 ml. ethanol gave a very small yield of crystals melting at 231-233°. Admixture with authentic pregnane-3α:20α-diol (m.p. 235-236°) depressed the melting point to 211-213°. On a different occasion, 50 mg. of pregnan-3α-ol-20-one gave 50 mg. of crude reduction product melting at 227-230° (corr.). No purification was attempted as the product was used for recovery experiments.

Reduction /

Reduction of neutral 17-ketosteroids with lithium aluminium hydride.

The reductions were conducted in a manner similar to that described for pregnanolone.

100 mg. androsterone gave 74 mg. of reduction product (but it was estimated that about 30% of the ethereal extract was lost). The product melted at 211-216°. After two recrystallizations from benzene, the melting point was raised to 225-225.5°. Androstane-3α:17β-diol melts at 223° and androstane-3α:17α-diol at 227-228°.[x]

103 mg. of androstane-3β-ol-17-one (epiandrosterone) gave 106 mg. of a product melting at 162-164°. After recrystallization from benzene the melting point was 166-167° and the yield 72 mg. The melting point of authentic androstane-3β,17β-diol is 168°.

103 mg. of $\Delta^5$-androsten-3β-ol-17-one (dehydroepiandrosterone) gave, on reduction, 100 mg. of a product melting at 176.5-179.5°. The melting point of authentic $\Delta^5$-androsten-3β,17β-diol is 182°.

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x The melting point figures quoted on this page are mostly taken from Gilman's Organic Chemistry(1943).

The method of estimating pregnanediol described by Sommerville, Gough and Marrian.

In order to save lengthy allusions to this method, its main features are here outlined briefly. A 24 hr. specimen of urine is diluted to 2.5 l. and duplicate 500 ml. aliquots taken for estimation. To each 500 ml. of urine, 100 ml. of toluene is added and the mixture brought to the boil under a reflux condenser. Concentrated HCl (50 ml.) is added and boiling continued for 10 min. The contents of the flask are cooled rapidly, transferred to a separating funnel, and the aqueous phase run off and extracted twice more with toluene. The combined toluene extracts are washed with NaOH solution and water, and taken to dryness. The residue forms the 'neutral fraction'.

For the precipitation procedure the neutral fraction is transferred to a conical centrifuge tube with ethanol and taken to dryness. The residue is dissolved in 4 ml. ethanol and precipitated by the slow addition of 16 ml. 0.1 N NaOH, the temperature being kept at 75°. The contents of the centrifuge tube are allowed to cool slowly in an incubator/

incubator at 37°. The precipitate is obtained by centrifugation, and the precipitation repeated twice using water instead of 0.1 N NaOH. The final, thrice precipitated product is then determined by the sulphuric acid colour reaction of Talbot, Berman, MacLachlan and Wolfe (1941).

Chromogenicity in the reaction with concentrated sulphuric acid.

Calibration curves have been constructed for the crude pregnanolone reduction product, the crystalline reduction product (presumably pregnane-3α:20β-diol), androstane-3α:17-diol and androstane-3β-17β-diol in the sulphuric acid colour reaction. The crystalline reduction product gives, within the experimental error, precisely the same surve as pregnane-3α:20α-diol; the crude reduction product is slightly less chromogenic, while the two androstane diols give only approximately one third of the colour given by the same amount of pregnanediol (Table 25).

Table 25 /

Table 25

Colour produced in sulphuric acid reaction by different compounds.

| | Spekker reading given by | | |
|---|---|---|---|
| | 0.1 mg. | 0.2 mg. | 0.4 mg. |
| Pregnane-3α:20α-diol | 0.098 | 0.206 | 0.425 |
| ?Pregnane-3α:20β-diol | 0.102 | 0.205 | - |
| Crude pregnanolone reduction product | 0.089 | 0.190 | 0.395 |
| Androstane-3α:17-diol | 0.033 | 0.065 | 0.133 |
| Androstane-3β:17-diol | 0.031 | 0.066 | 0.136 |

Recovery of androstane-3α:17-diol by the precipitation method.

Experiments were conducted to see whether androstane-3α:17-diol, the reduction product of androsterone, was eliminated by the precipitation method used. Table 26 shows the results obtained. It is assumed in the figures given as mg./24 hr. that each estimation would represent one-fifth of a 24 hr. specimen.

Table 26 /

Table 26.

Recovery of androstanediol after three precipitations.

| Androstanediol added. Mg./estimation | Androstanediol recovered Mg./estimation | Recovery % | Added androstanediol as mg. androsterone per 24 hr. | Recovery as pregnanediol Mg./24 hr. |
|---|---|---|---|---|
| 0.397 | 0; 0 | 0 | 2 | 0 |
| 0.793 | 0.060; 0.210 | 8; 27 | 4 | 0.1; 0.3 |
| 1.587 | 0.950 Mean of | 60 | 8 | 1.5 |
| 3.968 | 2.680 2 values | 68 | 20 | 4.4 |
| 7.936 | 6.140 | 77 | 40 | 10.2 |

Conclusion: At normal levels of androsterone excretion none or very little of its reduction product would appear to get through the purification process used for pregnanediol. In order to short-cut testing all the possible reduction products, it was decided to test the neutral fraction of normal male urine for apparent pregnanediol content after reduction with $LiAlH_4$

Reduction/

Reduction of neutral fraction of normal male urine with lithium aluminium hydride.

Two 24 hr. specimens of normal male urine were each made up to 2.5 l. and 1 l. duplicate aliquots were hydrolysed and extracted by the method described by Sommerville et al. (1948), but using double quantities throughout. The neutral fraction was in each case dissolved in 10 ml. of ethanol, 5 ml. of this solution was taken as the untreated control, while the remaining 5 ml. were taken to dryness, and the residue subjected to reduction with $LiAlH_4$. The reaction was conducted in a similar way to that described for pregnan-3α-ol-20-one, but 10 ml. of a 0.1 M solution of $LiAlH_4$ was used.

Table 27.

Apparent pregnanediol recovery from normal male urine (a) untreated and (b) following $LiAlH_4$ reduction.

| Specimen | Pregnanediol, mg. per 24 hr. | |
|---|---|---|
| | Untreated | Reduced |
| M1[x] | 0.185 | 0.050 |
| M2 | 0.100 | 0.035 |
| M2 | 0.100 | 0.020 |

x Duplicate lost.

Conclusion/

Conclusion: Reduction with $LiAlH_4$ does not appear to cause any increase in the apparent pregnanediol content of male urine blanks, but, on the contrary, a loss of material chromogenic in the sulphuric acid reaction is observed. The possibility still exists that although the various reduction products are not sufficiently insoluble to come through the precipitation procedure alone, they may be carried down with the pregnanediol precipitate. This could be tested for by adding pure pregnanediol to the reduced and untreated neutral fractions.

The reduction of pregnanolone by $LiAlH_4$ - small scale

Aliquots of a standard pregnanolone solution (containing 0.291 mg.) were taken to dryness and subjected to reduction with $LiAlH_4$ in the usual way. The product obtained by ether extraction was determined in the sulphuric acid colour reaction without further purification. Similar aliquots of the pregnanolone solution were also determined without reduction. At the same time aliquots of a standard pregnanediol solution were similarly treated. Duplicate reagent blanks, representing the whole reduction process were also determined, and the values/

values obtained after reduction corrected for the apparent pregnanediol content of the reagent blank.

Table 28

Recovery of 'apparent pregnanediol' following $LiAlH_4$ treatment of pregnan-3α-ol-20-one and pregnane-3α:20α-diol.

| Expt. | | Apparent pregnanediol content | | | Recovery % of theory |
|---|---|---|---|---|---|
| | | (a) Untreated mg. | (b) After reduction mg. | (b) corrected for reagent blank mg. | |
| 1 | Reagent blank | | 0.029<br>0.023 | | |
| | Pregnanediol (0.394 mg.) | 0.393<br>0.394 | 0.377<br>0.376 | 0.351<br>0.350 | 89<br>89 |
| | Pregnanolone (0.291 mg.) | 0.003<br>0.005 | 0.288<br>0.266 | 0.262<br>0.240 | (82<br>90) |
| 2 | Reagent blank | | 0.040<br>0.055 | | |
| | Pregnanediol (0.243 mg.) | 0.243<br>0.243 | 0.281<br>0.285 | 0.233<br>0.237 | 96<br>98 |
| | Pregnanolone (0.250 mg.) | 0<br>0 | 0.324<br>0.268 | 0.276<br>0.220 | 110<br>88 |

Conclusion/

Conclusion: Judged on the colour produced with sulphuric acid, in pure solution, pregnan-3α-ol-20-one appears to be almost quantitatively reduced by $LiAlH_4$. The reason for the fall in apparent pregnanediol content of the pregnanediol controls in one case is not known; if the pregnanolone reduction product suffered similar losses, the reduction certainly proceeded in a quantitative fashion in both experiments.

The reduction of pregnanolone by $LiAlH_4$ in the presence of neutral fraction.

The neutral fraction from two 24 hr. specimens of normal male urine was prepared. The apparent pregnanediol content of aliquots equivalent to one-fifth of a 24 hr. specimen was determined, both untreated and after $LiAlH_4$ reduction. To similar aliquots of the neutral fraction, known amounts of pregnanediol, or pregnanediol and pregnanolone were added, and the whole subjected to the reduction process. To check the efficiency of the reduction a control was also run in pure solution.

The/

The results are tabulated below (Table 29).

Table 29

Recovery of pregnanediol and pregnanolone reduction product after $LiAlH_4$ reduction in the presence of neutral fraction.

| Estimation | | Total pregnanediol recovered mg. | Pregnanediol recovered corrected for appropriate blank mg. | Recovery % of theory |
|---|---|---|---|---|
| 1 | Neutral fraction (untreated) | 0.045 | | |
| 2 | Neutral fraction (reduced) | 0.047 | | |
| 3 | Neutral fraction + 1.196 mg. pregnanediol | 0.890 | 0.845 | 71 |
| 4 | Neutral fraction + 1.196 mg. pregnanediol(reduced) | 0.850 | 0.803 | 67.5 |
| 5 | Neutral fraction + 1.196 mg. pregnanediol + 1.967 mg. pregnanolone | 2.180 | 2.135 | 67 |
| 6 | Pure solution, 1.196 mg. pregnanediol + 1.967 mg. pregnanolone | 2.225 | 2.198 | 69.5 |
| 7 | Reagent blank | 0.027 | | |

Conclusion/

Conclusion: While the recoveries of pregnanediol itself are very low in this experiment, the recoveries of pregnanediol + pregnanolone are no worse, suggesting that the reduction of pregnanolone proceeded quantitatively. The presence of neutral fraction did not reduce the efficiency of the reduction process, as shown by the almost identical recoveries obtained in estimations 5 and 6.

Recovery of pregnanolone reduction product after three precipitations.

A standard solution of the crude reduction product in ethanol was prepared. Aliquots were pipetted directly into conical centrifuge tubes and subjected to three precipitations, according to the directions of Sommerville et al. (1948). In experiments 1, 2 and 5 the normal cooling procedure was followed. In experiment 3, after the usual slow cooling in the incubator, the tubes were cooled to 0° before centrifuging. In experiment 4, the tubes were cooled slowly to 37°C., then cooled to room temperature, and finally to 0°.

Table 30 /

Table 30

Recovery of pregnanolone reduction product after three precipitations.

| Expt. | Reduction product added, mg. | Reduction product recovered mg. | Recovery % |
|---|---|---|---|
| 1. Normal method | 0.202<br>0.810<br>2.025 | 0.195; 0.162<br>0.702; 0.734<br>1.615 | 97; 80<br>87; 91<br>80 |
| 2. do. | 0.202<br>0.405<br>0.810 | 0.170; 0.205<br>0.295; 0.310<br>0.690; 0.616 | 85; 101<br>73; 76<br>85; 76 |
| 3. Cooled to 0° | 0.202<br>0.405<br>0.810 | 0.138<br>0.312; 0.315<br>0.672; 0.610 | 68<br>77; 78<br>83 75 |
| 4. do. | 0.202<br>0.405<br>0.809<br>1.621 | 0.134; 0.216<br>0.300<br>0.668<br>1.324; 1.092 | 66; 106<br>74<br>83<br>82; 67 |
| 5. Normal method | 0.683<br>1.366 | 0.388; 0.400<br>0.640; 0.736 | 57; 59<br>46; 54 |

Conclusion: The results obtained are very erratic, due to variable losses in the supernatant after centrifuging the precipitate. It was frequently observed that the precipitate did not spin down well, even with the addition of relatively large amounts of inert carrier (Hyflo supercel). In the case of the pregnanolone reduction product, the slow cooling precipitation does not appear to overcome this difficulty, nor does further cooling improve recoveries obtained.

Recovery/

Recovery of pregnanolone reduction product following modified precipitation procedures.

In an attempt to reduce the losses of pregnanolone reduction product which occur during the precipitation, the composition of the precipitation mixture was varied, both by increasing the ratio of water to ethanol, and by substituting methanol or acetone for ethanol. None of these modifications produced any improvement in the recoveries obtained (Table 31).

Table 31.

Recovery in modified precipitation procedures.

| Expt. | Pregnanolone reduction product added mg. | Precipitation mixture | No.of precipitations | Pregnanediol recovered mg. | Recovery % |
|---|---|---|---|---|---|
| 1 | 1.001 | 20% ethanol | 3 | 0.690<br>0.640 | 69<br>64 |
| | | 20% acetone | 3 | 0.735<br>0.645 | 74<br>65 |
| | | 15% ethan- | 3 | 0.625<br>0.640 | 63<br>64 |
| 2 | 0.397 | 20% methanol | 1 | 0.345<br>0.342 | 87<br>86 |
| | | do.,cooled to 0° | 1 | 0.308<br>0.317 | 78<br>80 |
| | | 20% acetone | 1 | 0.342<br>0.369 | 86<br>93 |
| | | do.,cooled to 0° | 1 | 0.306<br>0.333 | 77<br>84 |

Recovery of pregnanolone as pregnanediol after reduction and three precipitations.

Two lots of pregnane-3α-ol-20-one, A and B, one weighing 15.017 mg. and the other 12.014 mg. were reduced with $LiAlH_4$ in the usual way. The reduction product was in each case made up to 10 ml. in ethanol, and aliquots were taken for direct estimation, and for estimation after the precipitation procedure of Sommerville et al. (1948). The recoveries are shown in Table 32.

Table 32.

Recovery after reduction and three precipitations.

| Reduction | Reduction product added mg. pregnanolone | Pregnanediol found mg. | Recovery % of theory |
|---|---|---|---|
| A | | Before precipitation | |
| | 0.375 | 0.393 | 105 |
| | 0.751 | 0.777 | 104 |
| | | After precipitation | |
| | 0.751 | 0.520 | 69 |
| | 1.502 | 0.800 | 54 |
| | 3.003 | 2.40 | 80 |
| | 6.007 | 4.92 | 79 |
| B | | Before precipitation | |
| | 0.300 | 0.309 | 103 |
| | 0.601 | 0.622 | 103 |
| | | After precipitation | |
| | 0.601 | 0.330 | 55 |
| | 1.201 | 0.945 | 79 |
| | 3.003 | 2.34 | 78 |
| | 4.806 | 4.13 | 86 |

Conclusion: While the reduction with $LiAlH_4$ again appears to be quantitative, the recoveries following precipitation are poor at low levels, and are not invariably good at higher levels. Even when the reduction product added per estimation represents 3 mg. of pregnanolone, the recovery is only about 80%.

Discussion.

It has repeatedly been shown that the reduction of pregnanolone with lithium aluminium hydride, even on a small scale, gives almost quantitative yields of material which behaves like pregnanediol in the reaction with sulphuric acid. When the reduction product is subjected to the purification procedure of Sommerville et al. (1948), however, the losses are greater, and also more variable than those which occur with pregnane-3α:20α-diol. For this reason, the proposed method of estimating pregnanolones in urine has proved to be unsatisfactory. The possibility, which would always be present, of interference by the reduction products of other steroids of the neutral fraction, is a further disadvantage of the proposed method.

D. /

D. Summary.

1. A modification of the Zimmermann reaction suitable for estimating pregnan-3α-ol-20-one has been described.

2. A proposed method of estimating pregnanolone by reduction to pregnanediol with lithium aluminium hydride, and subsequent estimation of the increase in pregnanediol content, has been shown to be unsatisfactory.

## SECTION VI

## PRELIMINARY EXPERIMENTS ON THE HYDROLYSIS OF SODIUM PREGNANEDIOL GLUCURONIDATE BY OX-SPLEEN β-GLUCURONIDASE

A. Introduction.

B. Results.

C. Experimental.

D. Discussion.

E. Summary.

A. Introduction.

A number of steroids are excreted as glucuronides, as are some compounds of pharmacological interest, indoxyl, and certain phenols produced by bacterial action in the intestinal tract. Of the compounds known to be excreted as glucuronides, the steroids have most claim to be considered as normal constituents of the animal body. The fact that an enzyme, capable of hydrolysing specifically the β-glucuronide linkage, is widespread in animal tissues may thus be of considerable interest in connection with steroid metabolism, although the occurrence of glucuronic acid-containing polysaccharides must be remembered.

β-Glucuronidase was first described by Masamune (1934). It was originally supposed by Fishman (1939) that this enzyme possessed synthetic as well as hydrolytic activity. The work of Lipschitz and Bueding (1939) and Storey (1950) is not incompatible with this theory: these workers have shown that glucuronide formation, in vitro, does not occur by combination of the aglucone with glucuronic acid. The intervention of the same enzyme in the hydrolytic and synthetic actions is/

is therefore highly unlikely.

It has been evident for a number of years that there would be considerable advantages in possessing a simple and reliable method of carrying out enzymic hydrolysis of the steroid conjugates, since acid hydrolysis is known to alter the configuration and structure of some urinary steroids (e.g. Talbot, Ryan and Wolfe, 1943a) and to destroy others completely (Talbot and Eitingon, 1944). The enzymic method of hydrolysis not only offers the great advantage that it is unlikely to alter the structure of the compounds hydrolysed, but because of its specificity, the amount of non-steroid material liberated is greatly reduced. Extracts of enzyme-hydrolysed urine are almost colourless and contain far less contaminating material than extracts of the same urine which has been subjected to acid hydrolysis.

The use of an enzymic method of hydrolysis presents two disadvantages, one practical and one on theoretical grounds. Firstly, the difficulty in preparing large quantities of a stable enzyme preparation leads to a lack of uniformity in the preparations used. Secondly, the very specificity of the reaction may in some cases be a drawback, since/

since it is doubtful whether many steroids are excreted solely in combination with one acid residue. The evidence on this subject deserves to be referred to in greater detail.

The Specificity of Conjugation Processes.

So far, no urinary steroid has been isolated conjugated with any acid residue other than sulphuric acid or glucuronic acid, nor has there been any evidence suggestive that other types of conjugates are formed. This may possibly be due to the lability of other complexes under the isolation conditions used, rather than to their non-existence. In any event, large unexplored fields exist in this subject, which has never received systematic investigation.

Since no single steroid has been isolated both as a sulphate and as a glucuronide, there has been a tendency to believe that any particular steroid is conjugated either exclusively with glucuronic acid or exclusively with sulphuric acid. In recent years, considerable evidence has accumulated which shows that this is probably not the case.

Talbot et al. (1943a), studying methods of hydrolysis in connection with the estimation of urinary/

urinary neutral 17-ketosteroids, found that acid hydrolysis caused considerable destruction of dehydroepiandrosterone. They therefore devised an alternative method of hydrolysis involving boiling for one hour with barium chloride, the solution being buffered at pH 5.8; recovery of dehydroepiandrosterone from the added sulphate was over 90% by this method. The barium chloride hydrolysis method has been shown to liberate only 20-40% of the total 17-ketosteroids liberated by acid hydrolysis, in spite of the fact that there is no loss by destruction (Talbot et al., 1944; Bitman and Cohen, 1949). It is possible that the sulphates of other urinary neutral 17-ketosteroids are less easily hydrolysed than dehydroepiandrosterone sulphate; it is also possible that a proportion of these 17-ketosteroids are conjugated with other acids, such as glucuronic acid.

Talbot et al. (1944) showed that 20-30% of the total 17-ketosteroids were liberated by incubation with an acetone-dried powdered rat liver enzyme preparation which was active in hydrolysing sodium pregnanediol glucuronidate. Unfortunately the preparation was not tested for sulphatase activity, nor /

nor was the material not hydrolysed by the enzyme preparation subjected to subsequent barium chloride hydrolysis, so that, at the most, the results of this experiment can only be regarded as a tentative suggestion of the presence of neutral 17-ketosteroids as glucuronides.

The results of Buehler, Katzman, Doisy and Doisy (1949) are more definite. Using culture filtrates of Escherichia coli as a source of glucuronidase, which they claimed to be free from phenol sulphatase and alcoholic sulphatase, they showed that in the urine of normal men the glucuronidase liberated about 45% of the 17-ketosteroids liberated by acid hydrolysis. The corresponding figure for pregnancy urine was 70-90%. These results leave little room to doubt that at least some of the urinary 17-ketosteroids are conjugated both with glucuronic acid and with sulphuric acid.

The work of Cohen and Bates (1949) also suggests strongly that a single steroid may be conjugated in two forms. Using an enzyme preparation from Aspergillus oryzae which is claimed to contain a specific phenol sulphatase uncontaminated by an alcohol sulphatase or by a general phenol esterase, they/

they have compared the amounts of oestrogens (Kober positive material in the weak phenolic and strong phenolic fractions) liberated from human pregnancy urine by enzymic hydrolysis and by acid hydrolysis. If their results are correct, the percentage of the total oestrogens excreted which is present as the sulphate may vary greatly even in one subject and over a short period: the last month of pregnancy. For example, in one case, oestriol liberated by phenol sulphatase varied from 5% to 89% of the total oestriol liberated by acid hydrolysis. This suggests that, at least in late pregnancy where the daily excretion of steroids is very high, the nature of the steroid conjugate may be determined by the availability of one or other of the acids concerned, rather than by a predetermined tendency to be conjugated with a particular acid.

The results of Cohen and Bates might alternatively be explained by the presence of an inhibiting substance in varying amounts in the urine. In considering the results for the oestrone-oestradiol fraction this might seem a plausible explanation. It seems very unlikely, however, that approximately 90% of the oestriol is regularly excreted as the sulphate/

sulphate, and this leads us back to envisaging the interchangeability of the conjugating acids.

Observations made by Storey (1950) are of interest in this connection. During the course of an investigation into the mode of synthesis of glucuronic acid in mouse liver slices, he observed that the addition of sulphate ions had an inhibiting effect on glucuronide formation. The substrate in these experiments was o-aminophenol, which, in the rabbit, is conjugated to approximately the same degree to form both the ester sulphate and the glucuronide. Conjugation with sulphate was actually occurring, as was demonstrated by the increase in the ester phenol fraction, parallelling the fall in the glucuronide-conjugated phenol fraction.

Since the isolation of the steroid conjugates presents considerable difficulties, it is possible that the best method of ascertaining the nature of the conjugates of the different steroids is by the use of specific enzymic hydrolysis procedures. Whereas some steroids may be conjugated with one acid only, others may be excreted both as the glucuronide and the sulphate. Since there seems little/

little likelihood that the relative amounts of the two different conjugates, in such cases, will bear any relation to steroid metabolism, estimations of such compounds after one type of enzymic hydrolysis only would be of little value; but if the hydrolysis techniques were available, it would be quite possible, for research purposes, and in exceptionally interesting clinical cases, to carry out both types of enzymic hydrolysis.

The hydrolysis of steroid glucuronides with glucuronidase.

Fishman (1939) was the first to attempt the hydrolysis of steroid glucuronides by means of glucuronidase. He was able to show that oestriol glucuronide was readily hydrolysed by this enzyme, but he did not report any data on the efficiency of the hydrolytic procedure. Talbot, Ryan and Wolfe (1943b) used an acetone-dried rat liver glucuronidase preparation for hydrolysing 'sodium pregnanediol glucuronidate' isolated by the Venning (1937, 1938) method. The results of three experiments are given in which the pregnanediol recovered was 86, 88 and 92% of theory. Since Marrian and Gough (1946) have/

have shown that the maximum possible recovery of pregnanediol from such 'sodium pregnanediol glucuronidate' is less than 80% of theory, these results are obviously suspect.

Buehler et al. (1949) used a culture filtrate of E. coli as a source of glucuronidase for their experiments on urine. The bacteria were cultured in a medium containing menthol glucuronide to raise the glucuronidase activity. The preparation was claimed to hydrolyse menthol glucuronide 100% and oestriol glucuronide 95%, both under experimental conditions. Brooksbank and Haslewood (1950) also used a bacterial glucuronidase: their 'pregnanediol-like glucuronide' was hydrolysed by incubation with an active strain of Staphylococcus albus.

In the experiments described, a crude preparation of β-glucuronidase prepared from ox spleen has been used to hydrolyse 'sodium pregnanediol glucuronidate' in pure solution, added to male urine, and in pregnancy urine. In each case the amount of pregnanediol liberated has been compared with the amount liberated by acid hydrolysis, using the technique of Sommerville et al.(1948). 'Sodium pregnanediol glucuronidate' has been used as substrate in/

in these experiments because it is readily available, and the pregnanediol freed can be determined with a considerable degree of accuracy. It is hoped to apply the knowledge gained by this means to the problem of hydrolysing the glucuronides of more labile steroids.

B. Results:

In the earlier experiments, the conditions used to extract pregnanediol liberated after enzymic hydrolysis resembled as closely as possible those used after acid hydrolysis in the method of Sommerville et al. (1948). The recovery of pregnanediol was very variable, probably due to inefficiency in the extraction procedure. In later experiments, the hydrolysis mixture was extracted three times with an equal volume of ether. This eliminated all the emulsification which had been the difficulty when toluene was used as extracting solvent, and gave duplicate results which were in much better agreement.

The degree of hydrolysis obtained appears to vary even under apparently similar conditions, and it seems as if glucuronidase activity, measured by the amount of phenol liberated from phenol glucuronide under standard conditions, may not always truly represent the activity with respect to pregnanediol glucuronide. Another possible reason for the variability of the results might be a sudden loss of activity occurring in the enzyme preparations, although these are usually stable for at least a fortnight when stored in the refrigerator.

Since/

Since pure non-ketonic sodium pregnanediol glucuronidate was not available for these experiments, duplicate determinations of pregnanediol have been carried out after acid hydrolysis, using the method of Sommerville et al. (1948). At the levels of pregnanediol used, recoveries by this method should be of the order of 95-100% of theoretical. The pregnanediol found after acid hydrolysis is therefore considered as 100%, and the efficiency of enzymic hydrolysis is expressed in relation to this figure.

Most of the enzyme preparations appear to be comparatively insensitive to change in pH, under the conditions used, but the results suggest that pH 5 is nearer the optimum for the hydrolysis of sodium pregnanediol glucuronidate than pH 4. Fishman (1939) found that the optimum pH for the hydrolysis of oestriol glucuronide by ox spleen glucuronidase was 4.2.

The substrate concentrations used have been low in all cases, since it was desired to discover the degree of hydrolysis which could be attained in unconcentrated urine. According to Fishman (1939) the optimum substrate concentration for oestriol glucuronide/

glucuronide was $1.25 \times 10^{-3}$ M; the concentration of sodium pregnanediol glucuronidate used in the experiments described has been approximately one hundredth of this value. In pure solutions with substrate concentrations of the order of $1 \times 10^{-5}$ to $4 \times 10^{-5}$ M, and glucuronidase concentrations of approximately 10 glucuronidase units (G.U.) per ml., the pregnanediol liberated after 3 hr. at 37° has varied between 50 and 100% of that liberated by acid hydrolysis.

Pregnanediol can be recovered almost quantitatively from sodium pregnanediol glucuronidate added to male urine, although the presence of the urine does inhibit hydrolysis slightly. Recoveries of 88, 94 and 78% have been observed; in the case of the 88% and 78% recoveries, duplicate enzyme hydrolysis experiments in pure solution gave quantitative recoveries.

A few experiments to investigate the ability of glucuronidase to liberate pregnanediol from pregnancy urine have also been carried out. The pregnanediol found has represented 71, 74, 79 and 106% of the amount found after acid hydrolysis.

The method of extraction finally adopted is probably/

probably efficient for a large number of urinary steroids. The use of ether as extracting solvent has the additional advantage that it is easily removed at a low temperature. The whole procedure of hydrolysis and extraction reduces possibilities of destructive changes to a minimum.

C. Experimental:

Preparation of enzyme.

The glucuronidase was prepared by a simplified method based on the work of Graham (1946). The fresh ox spleen was weighed, minced, and dried by stirring with 2 volumes of dry acetone and filtering (i.e. for each 100 g. of spleen, 200 ml. of acetone was used). The acetone drying was repeated once, and the residue either spread out to dry directly, or dried by washing, on the Buchner funnel, with dry ether. The acetone-dried spleen was stirred for 1 hr. with 2 volumes of cold water. The aqueous extract was filtered through muslin, and then centrifuged, and the insoluble material discarded. The supernatant was adjusted to pH 5.0, M acetate buffer (pH 5.0, 50 ml./l.) was added, and the mixture was allowed to stand at 25°C. for 6 hr. (or sometimes at/

at room temperature for 15 hr.). The heavy precipitate which formed was removed by centrifugation and rejected. The clear supernatant was made 50% saturated with respect to $(NH_4)_2SO_4$ by addition of the solid, and allowed to stand 18 hr. in the refrigerator. The precipitate, containing the enzyme, was centrifuged down, dissolved in the minimum volume of water and dialysed against running tap-water for 15-24 hr. On some occasions, a further purification was obtained by rejecting the precipitate formed with 1.2 M $(NH_4)_2SO_4$ and then precipitating the enzyme with 1.8 M $(NH_4)_2SO_4$ (Mills, 1948).

Activity of enzyme.

The activity of the enzyme was determined by the method described by Kerr, Graham and Levvy (1948). To two 10 ml. centrifuge tubes containing 0.2 ml. of 0.1 M citrate buffer (pH 5.2), 0.2 ml. of 0.06 M phenol glucuronide (at pH 5.2) and 0.4 ml. of the enzyme preparation (appropriately diluted in some cases) were added. At the same time similar tubes made up to the same volume but containing no substrate (enzyme blank), no enzyme (substrate blank), and no substrate or enzyme (reagent blank) were also set up/

up. The tubes were kept for 1 hr. at 37°, timed from the addition of the enzyme, and the reaction stopped by the addition of 2 ml. of Folin-Ciocalteu reagent (B.D.H., diluted 1 in 5). The contents of the tubes were mixed, the protein precipitates were spun down, and a 2 ml. aliquot of the supernatant was pipetted into 4 ml. of 10% $Na_2CO_3$. The colour was developed at 37° for 20 min. and read against the reagent blanks in the Spekker photoelectric absorptiometer, using Ilford spectrum blue (602) filters. The reading was corrected for the enzyme and substrate blanks, and converted into µg. of phenol, by means of a phenol calibration curve constructed using the same experimental conditions. Hence the amount of phenol liberated by 1 ml. of the enzyme preparation in 1 hr., under the standard conditions was calculated, and the activity of the preparation expressed in glucuronidase units (G.U.) per ml., where 1 G.U. represents the amount of enzyme which will liberate 1 µg. of phenol from phenol glucuronide in one hour under the conditions described.

Estimation/

Estimation of pregnanediol.

In the control experiments, acid hydrolysis, extraction, purification and estimation of pregnanediol were carried out as described by Sommerville et al.(1948), but with the following differences: at least 12 hours were allowed to elapse between each precipitation, no charcoal was added before the final filtration, and the filter papers used were washed repeatedly with hot ethanol. After enzymic hydrolysis the pregnanediol was extracted with toluene in the earlier experiments, but later ether was found to be much more satisfactory. The pregnanediol in the neutral fractions obtained after enzymic hydrolysis often precipitated very slowly from aqueous ethanol, and for this reason each precipitation was left at 37° for 12-24 hours.

Results obtained using toluene to extract the pregnanediol.

(a) Hydrolysis of sodium pregnanediol glucuronidate in pure solution.

The enzyme and substrate concentrations, pH and time of incubation are shown in Table 33. The concentration of the substrate, sodium pregnanediol glucuronidate, is calculated from the amount of pregnanediol found after acid hydrolysis. The pH

was controlled by the addition of M acetate buffer to give a final concentration of 0.05-0.10 M, and the hydrolysis mixtures were in all cases incubated at 37° under a thick layer of toluene. The apparent pregnanediol content when the enzyme was incubated alone was estimated in each case, but these values have not been subtracted since they were of the same order as the normal reagent blanks.

Table 33 /

Table 33.

Recovery of pregnanediol from toluene extracts of glucuronidase hydrolysis mixture.

| Expt. no. | Notes on extraction procedure | pH | Time incubated hr. | Enzyme conc. G.U./ml. | Substrate conc. mg./l. | Substrate conc. $\times 10^{-5}$ M | Pregnanediol found. % of possible |
|---|---|---|---|---|---|---|---|
| 1 | Vigorous shaking | 5.0 | 48 | 9.6 | 11 | 2.1 | 99<br>99 |
| 2 | Filtered before extraction. Protein ppte. washed with toluene | 4.0<br>4.5<br>5.0<br>5.5 | 25 | 4.9 | 9.0 | 1.7 | 65<br>62<br>86<br>75 |
| 3 | Gentle shaking to avoid emulsions | 5.0 | 3<br>6<br>12<br>24 | 7.1 | 16 | 3.1 | 76<br>56<br>50<br>65 |
| 4 | | 5.0 | 2<br>6<br>11<br>24 | 10.0 | 19 | 3.6 | 69<br>51<br>68<br>54 |
| 5 | Brought to the boil at the end of the incubation period; filtered; filter washed with hot toluene | 5.0 | 3 | 9.7 | 4.9 | 1.0 | 103<br>74 |
| 6 | | 5.0 | 3 | 11.6 | 11.2 | 2.2 | 77 |
| 7 | | 5.0 | 3 | 9.8 | 8.8 | 1.7 | 56<br>36 |

Discussion/

Discussion: In the original experiment (1) ordinary extraction with toluene was used, without heating or filtration, and with normal shaking: emulsions were very bad and the extractions took two days to complete, but the recovery of pregnanediol was 99%. The erratic results obtained in experiments 3 and 4 indicate that gentle shaking is not effective in extracting pregnanediol quantitatively, even though the shaking at each extraction was continued for 2 minutes. These results are of interest, however, since they show that the greater proportion of the pregnanediol is liberated by incubation for 3 hours: under favourable conditions (expt. 5) complete hydrolysis may be obtained within this time. Experiments 5 and 6 suggested that improvement in recoveries might be obtained by boiling the hydrolysis mixture at the end of the incubation period, but experiment 7 shows that this is not the complete answer to the problem. The low recoveries in this case may have been partly due to the low activity of the enzyme preparation used: an unusually large amount of protein was added, probably causing increased losses on the protein precipitate.

(b) /

(b) Hydrolysis of sodium pregnanediol glucuronidate added to male urine.

Experiment 8:

To a one third aliquot of a 24 hr. specimen of male urine, measuring 550 ml., 35 ml. of M acetate buffer, pH 5.0, 5 ml. of a solution of 'NaPG', 15 ml. of glucuronidase solution and 100 ml. of toluene were added. At the same time an otherwise exactly similar hydrolysis mixture in which the urine was replaced by distilled water was also set up. After 8 hr., an additional 15 ml. of the enzyme preparation was added to each flask, and incubation continued for a further 16 hr. The activity of the enzyme preparation used in this experiment was not determined. The concentration of sodium pregnanediol glucuronidate in the hydrolysis mixture was $0.98 \times 10^{-5}$ M or 5.1 mg./l.

The hydrolysis mixture was extracted three times with 150 ml. portions of toluene, and the combined toluene extracts were washed twice with 100 ml. of N NaOH and twice with a similar amount of water.

The pregnanediol liberated by glucuronidase in the presence of male urine, and in pure solution, was/

was 88% and 98% respectively of that liberated by acid hydrolysis. No apparent pregnanediol was found when the urine or enzyme was incubated alone under similar conditions.

Experiment 9:

In this experiment 20.0 mg. of 'NaPG' was dissolved in water and added to a 24 hr. specimen of male urine, which was then made up to 2.5 l. Two 500 ml. aliquots were taken for acid hydrolysis, two for enzymic hydrolysis, and one was incubated without enzyme, to act as a control. The enzyme hydrolysis was allowed to proceed for 48 hr. after the addition to each 500 ml. of diluted urine, of 60 ml. M acetate buffer, pH 5.0, 80 ml. of enzyme solution and 100 ml. of toluene. The substrate concentration was 8 mg./l. or $1.55 \times 10^{-5}$ M, the enzyme concentration 14 G.U./ml. Extractions were carried out as in experiment 8. The results are given in Table 34.

Table 34 /

Table 34

Hydrolysis of 'NaPG' added to male urine.

| | Pregnanediol found mg. | $\frac{\text{Pregnanediol}}{\text{Pregnanediol after acid hydrolysis}} \times 100$ |
|---|---|---|
| Acid hydrolysis | 1.52<br>1.53 | 100 |
| Enzymic " | 1.41<br>1.48 | 93<br>96 |
| Urine + 'NaPG' alone | 0.09 | |
| Enzyme alone | 0.02 | |

(c) Enzymic hydrolysis of sodium pregnanediol glucuronidate in pregnancy urine.

Two experiments (10 and 11) on the hydrolysis of the pregnanediol complex in pregnancy urine were carried out, using the same extraction procedure as in experiment 8. In each case, 500 ml. of pregnancy urine was subjected to hydrolysis at pH 5.0. In another experiment (12) at the end of the incubation period the solution was made strongly alkaline by the addition of solid NaOH, the precipitated protein filtered off and the precipitate washed repeatedly with toluene. Extraction still caused very bad emulsification/

emulsification, and the results suggest that the recovery of pregnanediol was impaired by this procedure. The other conditions, and the efficiency of the hydrolysis are summarized in Table 35.

Table 35

Liberation of pregnanediol from human pregnancy urine.

| Expt. | Hr. incubated | Enzyme conc. G.U./ml. | Substrate conc. mg./l. | Substrate conc. $\times 10^{-5}$ M | Pregnanediol liberated (a) Acid hydrol. mg. | (b) Enzymic hydr. mg. | (c) By incubation alone mg. | $\frac{b}{a} \times 100$ % |
|---|---|---|---|---|---|---|---|---|
| 10 | 24 | 7.4 | 15 | 2.9 | 4.55 | 3.70<br>3.50 | 1.84 | 81<br>77 |
| 11 | 54 | 10.8 | 43 | 8.2 | 13.1 | 9.95<br>9.79 | 2.50 | 76<br>75 |
| 12 | 3<br>6<br>24 | 5.5 | 14 | 2.8 | 4.44 | 2.16<br>2.06<br>3.14 | | 49<br>46<br>71 |

Relative/

Relative efficiency of extraction with toluene and with ether.

Experiment 13.

'NaPG' equivalent to approximately 15 mg. of sodium pregnanediol glucuronidate was dissolved in 1200 ml. of water, and the solution divided into six 200 ml. portions. Two ( A and B) were subjected to acid hydrolysis. The remainder were incubated for 4 hr. with 40 ml. of enzyme solution, 20 ml. of M acetate buffer, pH 5.0, and 10 ml. of toluene. The enzyme concentration was 11.6 G.U./ml., and the substrate concentration 9.4 mg./l. or $1.83 \times 10^{-5}$ M. At the end of the incubation period, C and D were extracted 3 times with 250 ml. of ether, and E and F 3 times with 250 ml. of toluene, E being shaken vigorously, and F being shaken gently for 2 min. at each extraction. The results are shown in Table 36.

Table 36 /

Table 36

Extraction with ether and toluene.

| Estimation | Average Spekker reading | Aliquot | Pregnanediol found mg. | Pregnanediol found % of 'possible' |
|---|---|---|---|---|
| A | 0.231 | 1/10 | Average | |
| B | 0.211 | 1/10 | 1.52 | 100 |
| C | 0.179 | 1/10 | 1.22 | 80 |
| D | 0.181 | 1/10 | 1.25 | 82 |
| E | 0.180 | 1/10 | 1.24 | 82 |
| F | 0.133 | 1/10 | 0.92 | 60 |

Conclusion: Extracting three times with an equal volume of ether, or of toluene if shaken vigorously, yielded the same amount of pregnanediol and probably represents complete extraction of the pregnanediol present. Using ether the process is far less troublesome, as there are no emulsions to separate. Shaking gently with toluene is obviously not an efficient means of extracting pregnanediol.

Results/

Results obtained using ether to extract the pregnanediol liberated.

(a) The effect of pH on the hydrolysis of NaPG by glucuronidase.

Enzymic hydrolysis was carried out at pH 4.0, 5.0 and 5.5, with a substrate concentration of $2.33 \times 10^{-5}$ M. Half the enzyme was added at 0 hr. and the other half, two hours later; the total enzyme added gave a concentration of 17.8 G.U./ml. The results are summarized in Table 37.

Table 37.

| Expt. | Time incubated hr. | pH | (a) Pregnanediol found after acid hydrol. mg. | (b) Pregnanediol found after enzymic hydrol. mg. | $b/a \times 100$ % |
|---|---|---|---|---|---|
| 14 | 4 | 4.0 | 1.87,1.87 | 1.16 | 62 |
| | 4 | 5.0 | | 1.10, 1.17 | 59 63 |
| | 4 | 5.5 | | 1.17, 1.22 | 63 65 |
| | 24 | 5.0 | | 1.72, 1.73 | 92 93 |
| | 24 | 5.0 | | 0.03 | |

Conclusion /

Conclusion: From this experiment it would appear that the hydrolysis is not very sensitive to changes in pH, and that, on this occasion, 4 hours' incubation was insufficient to complete the hydrolysis, which was nearly complete in 24 hours.

(b) Experiments 15-17.

The composition of the 'NaPG' which had been used in previous experiments was uncertain; it had been subjected to a number of Girard separations and should have been pure sodium pregnanediol glucuronidate, but, probably owing to the use of insufficiently purified 'NaPG' as starting material, the final product was grossly contaminated and gave rise to only 66% of the theoretical amount of pregnanediol.

For the last three experiments (15-17) ordinary 'NaPG' prepared according to Venning's method, precipitated at least twice from acetone, and recrystallized once from 90% ethanol was used. (Found C, 62.3; H, 8.3; Na, 4.9%. Calculated for $C_{27}H_{43}O_8Na$: C, 62.5; H, 8.4; Na, 4.4%).

Experiment 15 /

Experiment 15. Hydrolysis of NaPG in pure solution

The results obtained in this experiment are shown in Table 38. The reaction mixtures were extracted three times with equal volumes of ether, washed twice with 1/7 volume of N NaOH and twice with water. The substrate concentration was in all cases $2.0 \times 10^{-5}$ M.

Table 38.

Hydrolysis of 'NaPG' in pure solution.

| Time incubated hr. | pH | Enzyme added G.U./ml. 0 hr. | 2 hr. | 4 hr. | (a) Mg.pregnanediol found after acid hydrol. | (b) Mg.pregnanediol found | $b/a \times 100$ % |
|---|---|---|---|---|---|---|---|
| 4 | 5.0 | 8.5 | 8.5 | | 1.62, 1.62 | 0.88, 0.78 | 54, 48 |
| " | 4.0 | 8.5 | 8.5 | | | 1.33 | 82 |
| " | 5.0 | 17.0 | | | | 1.20 | 74 |
| 24 | 5.0 | 8.5 | | 8.5 | | 1.36 | 84 |
| " | 5.0 | 17.0 | | 8.5 | | 1.62 | 100 |
| " | 5.0 | 8.5 | | | | 1.37 | 85 |

The figures obtained for the 4-hour hydrolysis at pH 5 in which the enzyme was added in two lots are unaccountably low. The higher figure obtained for hydrolysis at pH 4.0 is also unusual. With the

24/

24-hour hydrolysis it is interesting to note that whereas changing the enzyme concentration from 8.5 to 17 G.U./ml. had no effect on the final liberation of pregnanediol, raising the concentration to 25.5 G.U./ml. caused complete hydrolysis of the 'NaPG'.

Hydrolysis of 'NaPG' added to male urine

Experiment 16. 'NaPG' was subjected to acid hydrolysis in pure solution, and to enzymic hydrolysis both in pure solution, and when added to male urine. Hydrolysis mixtures, enzyme, substrate and urine blanks were set up as indicated in Table 39, and incubated for 24 hours. The substrate concentration was $2.25 \times 10^{-5}$ M.

Table 39 /

Table 39

Hydrolysis of NaPG added to male urine.

| Enzyme added G.U./ml. | | 'NaPG' soln. ml. | Water ml. | Urine ml. | M acetate buffer pH 4.85 ml. | Pregnanediol found mg. |
|---|---|---|---|---|---|---|
| 0 hr. | 4 hr. | | | | | |
| 7.7 | 7.7 | 10 | 0 | 200 | 20 | 1.44, 1.44 |
| 0 | 0 | 10 | 0 | 200 | 20 | 0.026 |
| 7.7 | 7.7 | 0 | 0 | 200 | 20 | 0.023 |
| 7.7 | 7.7 | 10 | 200 | 0 | 20 | 1.92 |
| 0 | 0 | 0 | 0 | 200 | 20 | 0.013 |
| 7.7 | 7.7 | 0 | 200 | 0 | 20 | 0.015 |
| 0 | 0 | 10 | 200 | 0 | 20 | 0.020 |
| Acid hydrolysis | | 10 | 200 | 0 | 0 | 1.83, 1.88 |

The enzyme hydrolysis figures have not been corrected for the substrate, urine and enzyme blanks, since these gave values of the same order as may be obtained by carrying the reagents alone through the estimation procedure. The enzymic hydrolysis in pure solution gave 1.92 mg. pregnanediol, as against 1.86 mg. liberated by acid hydrolysis, and 1.44 mg. hydrolysed enzymically in the presence of male urine, i.e./

i.e. enzymic hydrolysis in pure solution gave 103% of the pregnanediol found after acid hydrolysis, while if male urine was also present, the yield was 78%.

Liberation of pregnanediol in pregnancy urine by glucuronidase.

Experiment 17: Aliquots of pooled pregnancy urine were subjected to acid and enzymic hydrolysis. The enzymic hydrolysis was carried out at pH 4 and pH 5 for 24 hours. At the same time, to check the efficiency of the hydrolysis in the absence of urine, 'NaPG' in pure solution was subjected to the same hydrolysis conditions.

The glucuronidase preparation used had been prepared by concentrating a rather dilute preparation by $(NH_4)_2SO_4$ precipitation; it was expected to contain about 120 G.U./ml. giving a concentration in the hydrolysis mixtures of 22 G.U./ml. The activity was tested four days after it had been used, and was found to be only 17 G.U./ml., which would give a concentration of 2.5 G.U./ml. in the reaction mixture. The very low recoveries of pregnanediol obtained suggest that the second figure is more likely to have been correct.

200 Ml. of urine was incubated with 40 ml. of the appropriate M acetate buffer, 40 ml. of enzyme solution and 10 ml. of toluene. In the controls, 200 ml. of water containing 4.48 mg. of 'NaPG' was similarly treated. The amounts of pregnanediol found are shown in Table 40.

Table 40.

Hydrolysis of 'NaPG' in pure solution and in pregnancy urine.

| | pH | (a) Pregnanediol found mg. | (b) Pregnanediol found after acid hydrol. mg. | $a/b$ x 100 % |
|---|---|---|---|---|
| Pregnancy urine | 5.0 | 4.00 | 3.64, 3.86 | 106 |
| | 4.0 | 1.05 | | 28 |
| 'NaPG', pure solution | 5.0 | 0.64 | 2.02 | 32 |
| | 4.0 | 0.55 | | 27 |

These results are of unusual interest since, whereas the hydrolysis in pure solution only gave a low recovery of pregnanediol, the pregnancy urine incubated with the enzyme at pH 5.0 gave a result slightly higher than obtained on acid hydrolysis. The great effect of pH on the pregnanediol liberated from pregnancy urine is in marked contrast to the very slight effect caused in pure solution. Unfortunately no urine control was incubated without enzyme/

enzyme in this experiment, so it is impossible to say whether the results are due to contamination with highly active bacteria, and the bacterial enzyme being more sensitive to pH changes than the enzyme prepared from ox spleen, or to the presence in late pregnancy urine of compounds other than pregnanediol which are destroyed by acid hydrolysis, but which are estimated by the method of Sommerville et al. (1948). This question offers interesting possibilities for further research.

D. Discussion

While this work is only at a preliminary stage, the results obtained are of interest since they indicate the surprising efficiency of enzymic hydrolysis at very low concentrations of sodium pregnanediol glucuronidate. It is probable that the method of extracting pregnanediol now used is practically quantitative even when the hydrolysis mixture contains a large amount of protein material.

The variation in the activity of the different enzyme preparations is one of the striking, and disconcerting/

disconcerting, features of the problem. A means of determining rapidly the 'true' glucuronidase activity of each preparation, i.e. the activity with respect to sodium pregnanediol glucuronidate, is much needed. This situation underlies the difficulties inherent in the use of small batches of crude enzyme preparations for quantitative experiments.

It is hoped that future experiments will establish the optimum pH for the hydrolysis of sodium pregnanediol glucuronidate, and perhaps the relationship between the concentration of enzyme and the time required for complete hydrolysis. Further investigation of the effect of β-glucuronidase hydrolysis on the pregnanediol fraction isolated by the method of Sommerville et al. (1948) from human pregnancy urine is also needed.

## E. Summary

1. Crude preparations of β-glucuronidase have been prepared from ox spleen and used to hydrolyse sodium pregnanediol glucuronidate in pure solution, added to male urine and in pregnancy urine.

2. /

2. The pregnanediol liberated by enzymic hydrolysis in pure solution has ranged from 50 to 100% of that liberated by acid hydrolysis. The presence of male urine causes slight inhibition of this hydrolysis, but 78-96% of the pregnanediol has been liberated under these conditions.

3. Pregnanediol recovered from human pregnancy urine after incubation with β-glucuronidase has represented 71 to 106% of the pregnanediol estimated in the same urine after acid hydrolysis.

4. The results suggest that the optimum pH for this hydrolysis is nearer 5 than 4. In pure solution, complete hydrolysis of sodium pregnanediol glucuronidate may be achieved in three hours, under favourable conditions.

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